Soil microbial communities associated with two commercially important plant species indigenous to the fynbos region of South Africa: *Cyclopia* spp. (honeybush) and *Aspalathus linearis* (rooibos)

By

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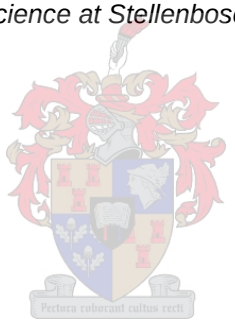

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Declaration

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Summary

The Cape Floristic Region (CFR) is characterized by nutrient poor soils which forged close symbiotic ties between plants and soil microorganisms for nutrient acquisition. *Aspalathus linearis* and *Cyclopia* spp. are two commercially important plant species endemic to the CFR in South Africa. These species are used to produce herbal teas known as rooibos and honeybush tea, respectively. Despite the important role soil microorganisms play in this environment, very few studies have been done to investigate the diversity and structure of the communities associated with these economically important plants. Furthermore, it is unclear what the effect of agricultural practices will be on these communities. Studies done on other agricultural systems, showed that the soil microbiome is greatly influenced by agricultural activities such as soil tillage, application of pesticides as well as monocropping systems. These activities often lead to the loss of soil productivity and biodiversity. We, therefore, hypothesised that the microbial communities associated with *A. linearis* and *Cyclopia* spp. plants will also be influenced by the respective agricultural activities. The overall aim of this study was to characterise and compare microbial communities associated with natural and commercially grown *A. linearis* and *Cyclopia* spp. plants. Furthermore, we aimed to investigated the effect different abiotic and physico-chemical factors may have on microbial communities in this unique region. Sampling was done on two occasions to include the dry, warm summer and the cold, wet winter seasons. A total of 29 bulk soil and 54 rhizosphere soil samples were collected during this study. The abiotic and physico-chemical properties of the soil samples were determined which included soil resistance, pH, total soil carbon, Na^+, K^+, nitrate and ammonia. Bacterial and fungal communities were characterised using next generation sequencing technology on the Ion Torrent (PGM) platform. For the bacteria, variable V4-V5 region of the 16S rRNA gene were amplified and sequenced. Fungal analysis used the internal transcribed spacer (ITS) region of the 18S rRNA gene. Bioinformatic and statistical analyses were performed using the software packages MOTHUR, PIPITS and R. No statistically significant differences were detected between bacterial communities from natural and commercial sites for both *A. linearis* and *Cyclopia* spp. plants. The plant-driven selection of rhizosphere microbiome for these two fynbos plants appeared to be very strong and was not significantly influenced by agricultural

activities. However, significant differences in bacterial communities were observed between samples collected during the different seasons. These seasonal changes support the contention that microbial taxa adapt and resist environmental changes differently. Furthermore, the overall taxonomic classification indicated that all soils were dominated by the bacterial orders Acidobacteriales and Actinomycetales. Both these groups are known to be dominant soil colonizing bacteria and are able to grow under low nutrient conditions, characteristic to the fynbos region. Additionally, the most dominant fungal phyla detected in samples included the Ascomycota and Basidiomycota. *Cyclopia* spp. samples were mostly dominated by the orders Agaricales, Chaetothyriales and Mortierellales, whereas *A. linearis* samples were dominated by the orders Chaetothyriales, Eurotiales and Helotiales. The β-diversity analysis showed that the *Cyclopia* spp. samples tended to cluster into commercial and natural groups. This might be due to the differences measured in soil pH between these two groups. Overall, little evidence was found to support our hypothesis. Bacterial communities from natural and commercial soil of both plant species were very similar and fungal communities associated with natural and commercially grown *A. linearis* plants also did not reveal any significant difference. However, fungal communities associated with natural and commercially grown *Cyclopia* spp. plants appeared to differ. In short, this study improved our knowledge on the biodiversity of soil microorganisms associated with two commercially important fynbos plant species and elucidated on factors that affected the microbial community structures.

Opsomming

Die Kaap Floristiese gebied word gekenmerk deur nutriënt arme grond wat die dryfveer is vir talle simbiotiese interaksies tussen fynbos plante en grond mikrobes. Twee kommersieël belangrike fynbos plante in die Kaap Floristiese gebied sluit in *Aspalathus linearis* en *Cyclopia* spp. Hierdie plante word gebruik vir die produksie van die welbekende rooibos en heuningbos kruie tees. Ten spyte van die belangrike rol wat grond mikroörganismes in hierdie omgewing speel, is daar nog relatief min studies gedoen om die diversiteit en struktuur van die mikrobiese gemeenskappe te beskryf. Dit is verder onduidelik wat die effek van landbou praktyke op die mikrobiese gemeenskappe is. Studies wat op ander landbou sisteme gedoen is, het bewys dat die grond mikrobes sterk beïnvloed word deur aktiwiteite soos grond bewerking, toediening van plaagdoders en monokultuur. Daar is bevind dat hierdie aktiwiteite grond produktiwiteit en die diversiteit van mikrobiese gemeenskappe verlaag. Gevolglik was die hipotese van hierdie studie dat die mikrobiese gemeenskappe wat met die grond van *A. linearis* en *Cyclopia* spp. plante geassosieer word, deur landbou aktiwiteite beïnvloed kan word. Die algehele doel van hierdie studie was om mikrobiese gemeenskappe wat geassosieër word met die grond van natuurlike en komersieël geplante *A. linearis* en *Cyclopia* spp. te beskryf en te vergelyk. Die effek wat verskillende abiotiese en fisies-chemiese faktore op hierdie gemeenskappe het, is ook ondersoek. Grondmonsters is op twee verskillende tye van die jaar versamel om beide, die droë warm somer en koue, nat winter seisoene in te sluit. ‘n Totaal van 29 omliggende en 54 wortelsfeer grond monsters is deur die loop van hierdie studie versamel. Verskillende abiotiese en fisies-chemiese faktore is bepaal en sluit in elektriese geleidings weerstand, pH, totale koolstof, Na^+, K^+, nitraat en ammoniak. Die samestelling van bakteriële en swam gemeenskappe is beskryf deur gebruik te maak van hoë omset volgordebepaling. Vir bakterieë is die veranderlike gebied V4-V5 van die 16S rRNA geen geamplifiseer. Swam analises het gebruik gemaak van die interne getranskribeerde spasie (ITS) gebied van die 18S rRNA geen. Bioinformatiese en statistiese analises is gedoen deur gebruik te maak van die sagteware pakkette MOTHUR, PIPITS en R. Geen statistiese beduidende verskille tussen bakteriële gemeenskappe wat geassosieer is met natuurlike en kommersieël geplante *A. linearis* en *Cyclopia* spp. is waargeneem nie. Die afleiding wat ons hieruit gemaak

het is dat die plant gedrewe seleksie van die wortelsfeer grond mikrobioom vir beide plant spesies baie sterk is en dat landbou aktiwiteit geen beduidende invloed op die gemeenskappe gehad het nie. Daar is wel verskille in die bakteriële gemeenskappe tussen die seisoene waargeneem. Hierdie seisoenale verandering in mikrobiese gemeenskappe ondersteun die feit dat mikrobiese taksa verskillend reageer en aanpas by verandering in omgewingstoestande. Verder het die taksonomiese klassifikasie van die bakterieë gewys dat die ordes Acidobacteriales en Actinomycetales grond monsters gedomineer het. Hierdie twee groepe is bekend daarvoor om verskeie grond omgewings te koloniseer, veral lae nutriënt omgewings wat kenmerkend is van die fynbos gebied. Die mees dominante swam filums wat waargeneem is in al die grond monsters sluit in die Ascomycota en Basidiomycota. Die *Cyclopia* spp. plant grondmonsters is gedomineer deur die orders Agaricales, Chaetothyriales en Mortierellales en *A. linearis* plante deur die orders Chaetothyriales, Eurotiales en Helotiales. Die β-diversiteit analises van *Cyclopia* spp. monsters was geneig om kommersiële en natuurlike monsters saam te groepeer. Dit is heel waarskynlik as gevolg van die verskille tussen grond pH tussen hierdie twee groepe. In geheel was daar min bewyse gevind wat die hipotese van hierdie studie ondersteun. Bakteriële gemeenskappe wat geassosieer is met beide natruurlike en kommersiële grond monsters was soortgelyk vir beide plant spesies, asook swam gemeenskappe wat geassosieer is met *A. linearis* plante. Daar is wel verskille waargeneem in swam gemeenskappe tussen natuurlike en kommersieël geplante *Cyclopia* spp. Kortom, hierdie studie verbeter ons kennis rakende die biodiversiteit van mikroorganismes wat geassosieër word met twee belangrike komersiële fynbos plante. Verder brei hierdie studie meer uit oor moontlike faktore wat mikrobiese gemeenskappe in fynbos grond kan beïnvloed.

Motivation

The Cape Floristic Region (CFR) is located in the south-western parts of South Africa and is recognized as one of the world's biodiversity hotspots (Cowling et al. 1996; Cowling et al. 2003). Endemic to this region is the commercially important herbal tea plant species, *Aspalathus linearis* (rooibos) and *Cyclopia* spp. (honeybush) (Joubert et al. 2008; McKay & Blumberg, 2007). Fynbos plants, including *A. linearis* and *Cyclopia* spp., are highly adapted to survive in this region which is characterized by nutrient-poor, acidic soil as well as warm, dry summers and cool, wet winters (Cowling et al. 1996; Lambers et al. 2011; Maseko & Dakora 2013). One of the most important factors in the successful adaption of fynbos plants is the symbiotic interactions with soil microorganism (Lambers et al. 2011; Maseko & Dakora, 2013). Despite the important role soil microorganisms play in this ecosystem, very little is known about their composition, structure and function. Only a few studies have been done on the microbial communities in the CFR and little is known of the interactions between microorganisms and fynbos plants (Beukes et al. 2013; Lemaire et al. 2015; Slabbert et al. 2010a; Slabbert et al. 2010b; Stafford et al. 2005; Visagie et al. 2009; Visagie & Jacobs, 2012). An important question is how microbial communities change during soil disturbances such as agricultural activities. However, very little is known about this. With this in mind, we decided to use these two commercially important fynbos plants to compare soil microbial diversity and structure of natural and disturbed fynbos systems. Apart from contributing to the biodiversity information of the fynbos biome, knowledge on microbial communities associated with endemic plants in natural environments, may yield information able to aid in the development of a more sustainable and profitable production of these plants.

The aim of this study, thus, was to explore soil microbial communities associated with natural and commercially grown *A. linearis* and *Cyclopia* spp. The first objective was to review the current knowledge on the fynbos biome, elaborate on soil functions and to emphasize the important role microorganisms play in soil ecosystems (Chapter 1). The second objective was to elucidate the structure and diversity of soil bacterial communities associated with two commercially important *Cyclopia* species, namely *C. subternata* and *C. longifolia* (Chapter 2). The third

objective explored the soil bacterial communities associated with *A. linearis* (Chapter 3). For both Chapter 2 and 3, bacterial communities associated with rhizosphere and bulk soil of natural as well as commercially grown plants were characterized. Furthermore, the effect of seasonal change on the bacterial communities was also investigated. The forth objective was to characterize and compare fungal communities associated with the rhizosphere soil of *Aspalathus linearis* and *Cyclopia* spp. collected during the wet seasons (Chapter 4). Lastly, this study highlighted some concluding remarks and future challenges associated with studying these complex soil ecosystems (Chapter 5).

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"Whatever you do, work at it with all you heart, as working for the Lord, not for men..."

Colossians 2:23

King James Bible

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Abbreviations

Anammox	Anaerobic ammonium oxidation
AM	Arbuscular mycorrhizal
amo	Ammonium monooxygenase
ARISA	Automated Ribosomal Intergenic Spacer Analysis
CFR	Cape Floristic Region
DGGE	Denaturing Gradient Gel Electrophoresis
EM	Ectomycorrhiza
Hao	Hydroxylamine oxidoreductase
$H_2PO_4^-$	Dihydrogen phosphate
$HP_2O_4^{2-}$	Hydrogen diphosphate
N	Nitrogen
NGS	Next Generation Sequencing
NH_2OH	Hydroxylamine
NH_4^+	Ammonia
NO_2^-	Nitrogen dioxide
NO_3^-	Nitrate
nxr	Nitrite oxidoreductase
P	Phosphorus
tRFLP	Terminal Restriction Length Polymorphism
WGS	Whole Genome Sequencing

Chapter 1

Microbial communities in fynbos soils: Structure, role and response to disturbances

1. Fynbos biome

The Cape Floristic Region (CFR) is endemic to the south-western parts of South Africa and is characterized by shrub- and heathland fynbos plants. This biodiversity hotspot is located in a Mediterranean climate region known for warm, dry summers and cool, wet winters (Cowling et al. 1996). Soil in the CFR is nutrient-poor, acidic and shapes the habitat for one of the world most diverse and endemic flora regions (Cowling et al. 2003; Richards et al. 1997; Van Wilgen et al. 2012). Fynbos plants are highly adapted to survive in this region and one of the most important factors contributing to their success is the symbiotic interactions with soil microorganisms (Elliott et al. 2007; Kanu & Dakora, 2012; Lambers et al. 2011; Maseko & Dakora 2013). The most significant advantage of these symbiotic interactions is the enhancement of nutrient acquisition for fynbos plants in the low nutrient soil (Maistry et al. 2013). Many studies were done to investigate specific bacterial (Cocks & Stock 2001; Dakora, 2012; Elliott et al. 2007; Gyaneshwar et al. 2011) and fungal (Allsopp & Stock 1992; Cloete et al. 2007; Cloete et al. 2009) symbiotic interactions with selected fynbos plants. However, very few studies focused on total microbial communities and factors that can alter their structure and/or function (Slabbert et al. 2010; Slabbert et al. 2014).

2. Fynbos soil structure and aggregation

One of the most important factors that determine the function of soil is its structure. Soil structure can be defined as the shape, size and arrangement of particles and pores (Bronick & Lal 2005; Osmond, 1993). Fynbos soil is mostly characterized as sandy and consists of particles between 0.05 mm and 2 mm in diameter. Soil is a habitat for a variety of prokaryotic and eukaryotic organisms such as bacteria, fungi, protozoa, nematodes, mites and plants roots (Dance, 2008; Mitchell et al. 1984). These organisms play a critical role in the formation, structure and stability of fynbos soil and directly affect soil aggregation that can be defined as groups of particles that bind together.

Plant roots play a central role in soil aggregation. Soil aggregation affects the movement of water and gasses through the soil (Dance, 2008). Physically, roots can

rearrange and entangle soil particles (Osmond, 1993). Furthermore, roots release exudates into the rhizosphere that alter the chemical and biological composition of the soil (Bais et al. 2006). Root exudates therefore, play a key role in determining the microbial composition of the rhizosphere and surrounding soil and these microbial associations with plant roots, as well as free living microorganisms, enhance soil stability (Zarnes et al. 2000). Soil particles can be bound together through extracellular compounds produced by bacteria and fungi. Additionally, fungal hyphae can entangle soil particles and often form hyphal networks, altering the physical properties of the soil (Klein & Paschke, 2004). Microbes therefore, play an important role in the structure of soil (Bronick & Lal, 2005). In return, the arrangement and size of particles affect the pore size, water retention, aeration and stability of soil, which directly affect soil microbial communities (Crawford et al. 2005).

3. Nutrients in fynbos soil

Soil nutrient gradients play an important role in biological activity and the distribution of plant species (Richards et al. 1997). Fynbos soil is characterized by very low nutrient concentrations, especially in terms of nitrogen (N) and phosphorus (P) content. Due to this characteristic of fynbos soil, most studies focussed on these two elements, although organic carbon and other micro-elements such as Na, K, Mg and Ca also play an important role in any ecosystem (Richards et al. 1997). Total N in fynbos soil ranges between 1 to 2 mgN/g and available P between 0.4 to 3.7 µgP/g (Cramer, 2010). Different factors can influence the N and P content in soil. In the fynbos biome, fire is one of the most important factors responsible for mobilizing nutrients in fynbos soil (Cramer, 2010). During fire events N can be transformed to organic forms (ammonia (NH_4^+) and nitrate (NO_3^-)) and organic P to orthophosphate (dihydrogen phosphate ($H_2PO_4^-$) and hydrogen diphosphate ($HP_2O_4^{2-}$)), making it available for plant uptake (Knicker, 2007).

4. Adaptations of plants living in the low nutrient fynbos soil

Despite the low nutrient content of soil in the CFR, this area is recognized as one of the world's biodiversity hotspot, with a very high diversity of vascular plants species

(Cowling et al. 2003; Myers et al. 2000; Van Wilgen et al. 2012). Plants from this region have undergone different adaptations in order to survive and flourish in these conditions (Elliott et al. 2007; Kanu & Dakora, 2012; Lambers et al. 2011; Maseko & Dakora 2013). These adaptations may involve several mechanisms to improve the uptake of both N and P which play a critical role in plant development through their importance in the synthesis of proteins, nucleic acids, secondary metabolic products, coenzymes and phospholipids (Lambers et al. 2006; Miller & Cramer, 2004).

One way in which plants adapted to maximize the acquisition of P, is through specialized structures known as root clusters (Lambers et al. 2011). These bottle brush-like structures are usually present on primary or secondary lateral roots and assist in the mobilization of P in the soil (Skene, 1998). Phosphorus is highly immobile and very little reaches plant roots through mass flow or diffusion (Lambers et al. 2006; Mitchell et al. 1984). The root clusters increase the surface area of the roots improving contact with P in the soil (Lambers et al. 2006). In addition, root clusters mobilize organic and inorganic P through the exudation of carboxylates (Power et al. 2010). Solubilisation of P in soil is also aided through the production of phosphatases in the rhizosphere by free living and symbiotic microorganisms (Vance et al. 2003). This and other symbiotic interactions with microorganisms are a vital mechanism allowing plants to live in the low nutrient fynbos soil.

Many of the plant-microbe symbioses occur in the rhizosphere, the narrow zone of soil surrounding plant roots, and are affected by the presence of plant roots (Cloete et al. 2007; Doornbos et al. 2011; Maseko & Dakora, 2013; Masson-Boivin et al. 2009). Plants are able to communicate with microorganisms present in the soil through root exudates (Bais et al. 2004). These root exudates are likely to shift the soil microbial community towards species that can effectively compete for the available resources and also form symbiotic associations with the plant (Dennis et al. 2010).

One of the best studied symbiosis examples is the mutualism between mycorrhizal fungi and fynbos plants which is an important adaptation mechanism to the low soil P concentrations (Lambers et al. 2006). Two of the most widespread mycorrhizal types found in the fynbos biome, include species from the arbuscular and

ectomycorrhizae (Cloete et al. 2007; Maseko & Dakora, 2013). Roots of the host plant provide the fungi with a carbon (C) source and in return the fungi increase P-acquisition as much as three to five times for the plant (Cloete et al. 2007). Mycorrhizal hyphae are able to scavenge larger volumes of soil for nutrients, can penetrate smaller soil pores than plant roots and increase the surface area for P uptake (Marschner & Dell, 1994). After uptake and translocation of P from the soil by the hyphae, high-affinity protein transporters transfer P to the cortical cells in the plant roots. Although this translocation of P from the soil to the plant requires metabolic energy, it overcomes the slow diffusion of direct uptake through the plant roots (Smith et al. 2011).

Symbiosis between plants and bacteria also play a critical role in the low nutrient fynbos soil, particularly in N acquisition (Lemaire et al. 2015). The second most species rich plant family in the CFR region, the Leguminosae (Fabaceae), consists of numerous genera including two commercially important plants, *Aspalathus* and *Cyclopia* (Kanu & Dakora, 2012; Lemaire et al. 2015). Leguminous plants can form specialized plant organs known as root nodules, which is induced by nitrogen-fixing bacteria (rhizobia). These bacteria fix atmospheric nitrogen to ammonia through nitrogenase enzymes (Doornbos et al. 2011; Masson-Boivin et al. 2009). The most prominent rhizobial species detected in fynbos legume plants belongs to the genus *Burkholderia.* Species from this beta-rhizobia group are known to be well adapted and tolerant of the acidic soil of this region (Lemaire et al. 2015).

Root nodules are primarily formed under nitrogen-limited conditions which is often the case in fynbos soil. Their formation is initiated by root exudation of secondary metabolite signalling molecules (Dakora & Phillips 2002; Haichar et al. 2014). Flavonoids are the most prominent, diverse and widely studied secondary metabolites released by plants roots (Subramanian et al. 2007). More than 4000 different flavonoids have been identified in vascular plants and they are all synthesized via the phenylpropanoid pathway (Haichar et al. 2014).

These secondary metabolite signalling molecules induce the expression of bacterial *Nod* genes and the synthesis and transport of Nod factors, low molecular β,1-4-linked *N*-acetyl glucosamine compounds (Dakora & Phillips, 2002; Haichar et al.

2014; Stougaard, 2000). Subsequently, the Nod factors induce the initial response of forming pre-infection threads, root cortical cell division and curling of root hairs (Hassan & Mathesius, 2012). This two-way communication between a legume host and rhizobium is often very specific. Nodule formation will only be initiated if the specific signal molecule induces the *Nod* gene activity of the rhizobium. Furthermore, if the bacterium produces Nod factors which is not recognized by the specific plant, nodulation will not occur (Stougaard, 2000). Host specificity is not only determined by the chemical communication between plant and rhizobium. It was also shown that environmental factors such as soil acidity are important ecological drivers of specificity (Lemaire et al. 2015).

5. Biogeochemical cycling

Geochemical cycling constantly supplies and removes products, preventing the depletion of substrates on earth. These cycling processes can be divided into two major categories, namely abiotic geochemical cycling which is based on acid/base chemistry, and biogeochemical cycling based on redox reactions (Falkowski et al. 2008). Known as the engineers of life, microorganisms play a central role in biogeochemical cycling (Cotner & Biddanda, 2002; Falkowski et al. 2008). In the CFR, biogeochemical cycling of N and P is of great importance because not only are these elements important to form many biological macromolecules essential for life in soil (Lambers et al. 2006; Miller & Cramer, 2004), but are also limiting growth factors in this region (Cramer, 2010; Knicker, 2007). Therefore, optimal cycling of N and P in the fynbos soil is one of the key factors that contribute to the successes of the high plant diversity (Figure 1.1 and Figure 1.2).

5.1 Nitrogen

Plants are unable to assimilate nitrogen (N_2) gas, due to the strong triple bond between the two diatomic nitrogen atoms making it inert (Shridhar, 2012). Therefore, plants depend on dissolved forms of inorganic nitrogen, mainly NH_4^+ and NO_3^- (Pate, 1973). However, plants are also able to assimilate organic nitrogen in the form of amino acids (Näsholm et al. 2009). Consequently, N_2 needs to be reduced to inorganic forms in order to be available for plant uptake and this is done

primarily by soil microorganisms. Through nitrogen fixation, atmospheric N_2 can be reduced to NH_4^+ by bacterial species such as *Burkholderia*, *Frankia* and *Rhizobium* (Canfield et al. 2010; Franche et al. 2008). Under anaerobic conditions this reaction is catalyzed by the nitrogenase enzyme complex (Peters & Szilagyi, 2006; Rees & Howard, 2000). The molybdenum-containing nitrogenase is characterized the best. It consists of two subunits, an iron containing dinitrogenase reductase (Fe protein) and a molybdenum iron dinitrogenase (MoFe protein). These proteins are encoded by the highly conserved nifH and nifDK genes, respectively (Penton et al. 2013; Shridhar, 2012). Other than nitrogen fixation, NH_4^+ can also be reintroduced into the environment when organisms die (Canfield et al. 2010).

Under aerobic conditions, the released NH_4^+ can be oxidized to hydroxylamine (NH_2OH), nitrogen dioxide (NO_2^-) and ultimately NO_3^- which can be assimilated by various organism and plants. Nitrifying bacteria responsible for this reaction include the bacterial genera *Nitrobacter* and *Nitrosomonas* (Mobarry et al. 1996). Nitrification is catalyzed by three separate enzymes namely ammonium monooxygenase (*amo*), hydroxylamine oxidoreductase (*hao*) and nitrite oxidoreductase (*nxr*) (Canfield et al. 2010).

Under anaerobic conditions some microorganisms are able to use NO_3^- as an electron acceptor in the oxidation of organic carbon. Nitrate reduction produces either N_2 gas through denitrification or NH_4^+ through dissimilatory nitrate reduction (Smith & Tiedje, 1978). Four different reductase enzymes are involved in catalysing these reactions namely dissimilatory nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductases. These reductase enzymes are encoded by various genes including *nas, euk-nr, narG, napA, nir, nrf, norB, hao* (Canfield et al. 2010). Nitrogen gas can also be released in the atmosphere through a process known as anaerobic ammonium oxidation (anammox). This process is performed by a group of bacteria known as the Planctomycetes which normally occurs in marine environments (Damsté et al. 2005). However, this group of bacteria was also found to be present in a wide variety of soil environments (Humbert et al. 2010).

5.2 Phosphorus

In contrast to the N cycle, P has no interchange with the atmosphere and is therefore, an open/sedimentary cycle (Rodríguez & Fraga, 1999). The majority of soil P is insoluble and the concentration of soluble P in the soil, especially in the fynbos, is very low (Cramer, 2010). Soil P can occur in organic or inorganic forms (Sharpley, 1995). However, plants are only able to assimilate inorganic P in the form of orthophosphates, $H_2PO_4^-$ and $HP_2O_4^{2-}$ (Knicker, 2007).

Organic soil P is mainly in the form of phytate (inosito phosphate), but can also be phosphomonoester, phosphotriesters or phosphodiesters (phospholipids and nucleic acids) (Rodríguez & Fraga, 1999). In order to be available for plant uptake, these substrates must first be hydrolyzed to inorganic P. This mineralization of organic substrates is carried out by phosphatase enzymes, secreted by plant roots as well as microorganisms, that hydrolyze phosphate esters (Dakora & Phillips, 2002; Maseko & Dakora, 2013; Olander & Vitousek, 2000).

Rock phosphate is the most abundant form of inorganic P in nature (Azcon et al. 1975). This highly insoluble form of P is mainly represented by mineral complexes such as apatite, oxyapatite and hydroxyapatite. Furthermore, poorly soluble mineral phosphate can also be associated with hydrated oxides of Fe, Al and Ca. Phosphate-solubilizing bacteria are usually more abundant in the rhizosphere (Rodríguez & Fraga, 1999). Commonly occurring soil phosphate solubilizers include members form the genera *Pseudomonas* and *Bacillus*. These bacteria are able to solubilise inorganic P mineral complexes through the production of various organic acids (Rodríguez et al. 2006).

6. Soil microbial abundance, diversity and function

Microorganisms play a central role in soil ecosystem functioning through biogeochemical cycling (Cotner & Biddanda, 2002; Falkowski et al. 2008). The abundance of soil microorganisms is much greater than that of any other eukaryotic organisms in soil ecosystems (Dance, 2008). It has been estimated that one gram soil may contain as many as 10^{10} – 10^{11} bacterial cells and up to 200 m of fungal

hyphae which greatly contribute to the microbial biomass in soil (Gupta & Germida, 1988; Van Der Heijden et al. 2008).

Soil microorganisms are not only abundant, but also highly diverse. Microbial diversity can be defined as the relative abundance of different species in a community (Nannipieri et al. 2003). In soil environments, nutritional and abiotic conditions often differ within micrometers, which often lead to the high heterogeneity of microbial communities in soil (Franklin & Mills, 2003; Torsvik & Øvreås 2002; Veen & Elsas, 2004). Apart from the abiotic properties of soil, various biological mechanisms can influence soil microbiomes. For instance, it is well known that bacterial communities in rhizosphere soil are less diverse but greater in abundance compared to bulk soil (Dennis et al. 2010; Smalla et al. 2001; Uroz et al. 2010). In the rhizosphere, root exudates create selective conditions for a specific microbial community. These exudates serve as communication signals and nutrient sources to microorganisms which lead to an increase in abundance of microbes that are able to utilize these nutrients (Bais et al. 2004). As a result, less diverse communities are often present in rhizosphere soil (Dennis et al. 2010). Furthermore, it was found that the abundance and activity of microorganisms increased with an increasing plant diversity (Teinauer et al. 2015). It has been suggested that relationships between plants and microbial communities can lead to a higher microbial diversity in areas with more diverse plant communities (Zak et al. 2003). However, Prober and co-workers (2015) showed that plant diversity only predicts the beta diversity (diversity over space and time) but not the alpha diversity (diversity within a specific area) of the microbial communities.

It is hypothesized that there is a strong link between microbial diversity and soil function (Muller et al. 2002). However, to study this link is challenging due to the complexity of soil. With the help of molecular techniques such as next-generation sequencing (NGS), together with various bioinformatics tools, scientists are beginning to understand some of the complex links between microbial diversity and function. By using these techniques, it was found that higher microbial diversity increase resistance and resilience of soil processes, consequently communities are more resilient and adaptable to environmental change (Girvan et al. 2005; Mendes et al. 2015; Nannipieri et al. 2003). This is most likely due to higher functional

redundancy of more diverse communities. Functional redundancy can be defined as the ability of a microbial taxon to perform a specific function at the same rate and environmental conditions as another taxon (Allison & Martiny, 2008).

7. Soil microbial community composition

Soil microbial species that are pervasive are most likely to flourish and tolerate a variety of different environmental conditions (Barberán et al. 2014). These species are known as copiotrophs or r-strategists and are usually fast growing and abundant in favourable conditions. However, these species are often sensitive to environmental stress (Minz et al. 2013). In contrast to copiotrophs, slow growing oligotrophs (k-strategists) can persist under unfavourable conditions such as low nutrient levels (Barberán et al. 2014). Although often outcompeted by copiotrophs in favorable conditions, these species are able to remain viable under stressful conditions (Fierer et al. 2007). Oligotrophs often utilize low molecular weight substrates that occur in very low concentrations within the environment (Eilers et al. 2010; Semenov, 1991).

Characterizing the structure of soil microbial communities is very complex due to the heterogeneous nature of soil, together with the many factors that can have an influence on the microorganisms (Franklin & Mills, 2003). Although the structure of soil microbiota differs greatly over soil type, location, environmental factors and time, some microbial phyla have been found to dominate in these environments.

7.1 Bacteria

Actinobacteria is one of the most dominant bacterial phyla in soil environments (Schrempf, 2013). Within the Actinobacteria, the Gram positive, spore forming mycelial actinomycetes are known to produce secondary metabolites, hormones, siderophores, antibiotics and enzymes. They can also solubilise nutrients that can be beneficial to plant growth (Norovsuren et al. 2007; Qin et al. 2015; Schrempf, 2013). Furthermore, actinomycetes are known to be able to survive in dry soil due to their ability to form spores (Williams et al. 1972). Some actinomycetes are also known to have a very low growth rate and are able to grow under low nutrient levels;

consequently, they are very persistent in soil environments. However, according to a study done by Fierer and co-workers (2007), Actinobacteria could not be assigned to either copiotrophic or oligotrophic categories based on changes in soil C availability. Some of the most frequent isolated actinobacterium species include *Agromyces*, *Arthrobacter*, *Corynebacterium*, *Frankia, Micobacterium* and *Streptomyces* (Davies & Williams, 1970; Franche et al. 2008).

Another dominant soil bacterial phylum is the Acidobacteria (Mendes et al. 2015; Quaiser et al. 2003; Ward et al. 2009). Generally oligotrophic (Fierer et al. 2007), the majority of Acidobacteria is unculturable. However, isolates that have been cultured grow very slow on complex, low-nutrient media (Ward et al. 2009). Some of the few described species include *Acidobacterium capsulatum*, *Geothrix fermentas* and *Holophaga foetida* (Quaiser et al. 2003). Due to the difficulties associated with culturing these species, very little is known about their habitat, physiological characteristics, potential functions and metabolic contributions to the environment (Fierer et al. 2007; Ward et al. 2009). In soil environments, Acidobacteria are mainly detected by molecular methods using 16S rDNA sequencing. Although this molecular method is useful to characterize members of this phylum, no physiological or phenotypic characteristics can be determined (Quaiser et al. 2003).

The Proteobacteria is another dominant bacterial phylum detected in most soil environments (Fierer et al. 2007). Bacteria of this phylum play an important role in carbon and nitrogen cycling and cover a vast range of metabolic, morphological and physiological diversity (Spain et al. 2009). Proteobacteria is divided into five subclasses namely α, β, γ, δ and ε. From all the subclasses, the α- and β-Proteobacteria are the most commonly detected in soil environments. Various plant symbiotic genera, known to play an important role in nitrogen fixing, belong to the α-Proteobacteria and includes species such as *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium*. Other non-symbiotic genera include *Blastochloris* and *Chelatococcus* (Stepkowski et al. 2003). Species from β-Proteobacteria are generally copiotrophic (Fierer et al. 2007). As a result, members of the β-Proteobacteria are often more abundant in rhizosphere soil which consists of higher C levels compared to bulk soil (Mendes et al. 2015). Nitrifying bacteria that belongs to the genus *Nitrosomonas* and *Nitrosospira* as well the nitrogen fixing *Burkholderia* that are often abundant in

fynbos rhizosphere soil, all belong to the subclass β-Proteobacteria (Fierer et al. 2007).

7.2 Fungi

The fungal kingdom is the most diverse eukaryotic group of organisms in soil environments and includes macroscopic, filamentous and unicellular morphological forms (Tedersoo et al. 2014). This group often has a higher biomass and metabolic activity than bacteria and also plays an important role in nutrient cycling and plant health. Filamentous fungi are able to form hyphal networks which can stretch centimetres or in some cases even meters through the soil (Klein & Paschke, 2004). These networks serve as communication pathways to microbe-microbe and microbe-plants interactions (Barto et al. 2012). Conversely, unicellular fungi known as yeasts, are distributed unevenly in soil environments and are often more abundant in rhizosphere soil, especially close to fruit bearing plants (Botha, 2011).

Two of the best characterized and commonly found fungal phyla within soil environments include members from the Ascomycota and Basidiomycota. The Ascomycota is the largest and most diverse fungal phylum and consist of approximately 64 000 known species. This phylum is characterized by the sac-like structure known as the ascus in which ascospores (meiotic) are produced (Schoch et al. 2009). Frequently isolated filamentous fungi include *Alternaria, Aspergillus*, *Fusarium, Penicillium,* and *Trichoderma* (Vinale et al. 2008; Visagie et al. 2014). Common soil colonising yeast genera from this phylum includes *Candida, Geotrichum,* and *Saccharomyces* (Botha, 2011).

The second largest fungal phylum, the Basidiomycota, is one of the most important decomposer groups in soil environments (Lynch & Thorn, 2006). Various saprotrophic fungi are included in this phylum and are able to decompose a number of recalcitrant compounds. Many species in this phylum are lignocellulose-degrading saprotrophs, such as *Phanerochaete chrysosporium* and *Trametes versicolor*, which produce a number of lignin-modifying enzymes (Lynch & Thorn, 2006; Thorn et al. 1996). Basidiomycetous fungi also contain a number of macroscopic ectomycorhizal fungi, such as *Amanita, Boletus* and *Lactarius*, that are known to form mutualistic

associations with plant roots (Kreuzinger et al. 1996). However, plant pathogens such as *Rhizoctonia,* are also included in this phylum (Lynch & Thorn, 2006). Furthermore, studies showed that some basidiomycetous yeasts, such as *Cryptococcus* and *Dioszegia*, were found to be associated with arbuscular mycorrhizal (AM) roots (Renker et al. 2004). The co-occurrence of yeasts and AM fungi were shown to enhance the colonization and growth of plant roots (Alonso et al. 2008). Arbuscular mycorrhizal fungi are obligate symbionts of vascular plants and play an important role in plant growth and survival (St-Arnaud et al. 1996). This group of fungi are included in the phylum Glomeromycota and the largest genus is *Glomus* (Schwarzott et al. 2001). Other common soil born genera, *Mucor* and *Rhizopus*, are included in the Zygomycota (Mouhamadou et al. 2013).

8. Physico-chemical factors influencing microbial soil habitat

Many studies have demonstrated the strong correlation between microbial community structure and the physical and chemical properties of soil (Lauber et al. 2008; Marschner et al. 2001; Wardle et al. 2004). Different abiotic and physico-chemical factors can affect microbial communities. Some of these factors which also play an important role in the ecosystem functioning of the fynbos biome, include pH levels (Lauber et al. 2009; Rousk et al. 2010), variable soil temperatures (Smit et al. 2001; Sheik et al. 2011; Stevenson et al. 2014), water availability (Drenovsky et al. 2010; Kavamura et al. 2013) and soil aeration (Canfield et al. 2010).

In general, soil bacterial communities strongly correlate with changes in soil pH due to their narrow pH range for optimal growth (Fierer & Jackson, 2006; Rousk et al. 2010). As suggested by Lauber and co-workers (2009), bacterial diversity and community structures are to some point even predictable when considering soil pH (Lauber et al. 2009; Stopnisek et al. 2014). Changes in soil pH can directly affect bacterial communities by causing physiological constraints. Soil pH that falls outside the tolerance levels of certain groups of bacteria will reduce or eliminate the growth of these groups since they are unable to survive at that specific pH range (Rousk et al. 2010). Additionally, pH can also indirectly affect bacterial community structures since pH plays a central role in nutrient availability (Miller & Cramer, 2004), metal solubility (Masscheleyn, 1991) and salinity (Lauber et al. 2009) in soil environments.

The pH of fynbos soil is naturally low (between 4.5 – 5.5) and it is, therefore, expected to play a major role in shaping the microbial community structure. Some bacterial species commonly detected in fynbos soil, such as *Burkholderia* spp., are even known to be acid tolerant (Stopnisek et al. 2014). In contrast to the high predictability of bacterial communities, fungi are mostly unaffected by soil pH due to their wide tolerance range (Rousk et al. 2010).

Microbial structure and activity can also be influenced by soil temperature. It is well known that temperature plays an important role in determining the rates of all physical, chemical and physiological reactions (Pietikäinen et al. 2005). Therefore, changes in soil temperature often lead to a change in microbial structure and activity. Bacteria usually favour warmer soil temperatures, whereas fungi prefer colder temperatures (Lipson et al. 2002). Some microbes are able to adapt to changes in soil temperatures through the production of extracellular enzymes (Stark et al. 2015). These microbes will often out-compete other microbes unable to adapt to these changes. Two of the major causes for temperature changes in fynbos soil environments include seasonal changes (Lipson et al. 2002; Smit et al. 2001; Stevenson et al. 2014) and fires (Bond et al. 2003).

Seasonal changes not only affect soil temperature, but also the water content of soil environments, which indirectly affect soil aeration (Osmond, 1993). Water is responsible for the movement of ions, nutrients, gases and heat as well as transporting soil biota such as bacteria, protozoa and nematodes (Abu-ashour et al. 1994). After heavy rains, soil pores are filled with water and become saturated. The saturated pores directly affect the aeration status of the soil and in turn affect the microbial community structure. The effect of soil water availability on microbial communities is still not yet fully understood. In two different studies, contradictory results were obtained during the evaluation of the effect of water on different microbial communities. In a study done by Drenovsky and co-workers (2010) soil samples were analyzed from different agricultural land-use types including grape, peach, rice, fig and bean to name a few. Using phospholipid fatty acid analysis, they found that dry soils tend favour Gram-negative bacteria and fungi, whereas wetter soils tend to favour Gram-positive and anaerobic bacteria. In contrast to these results, Kavamura and co-workers (2013) sampled soil from a semi-arid region.

Using high throughput sequencing they show that dry soils favour Gram-positive bacteria such as *Bacillus* and Actinobacteria and wetter soils favour Gram-negative bacteria especially groups such as Bacteriodetes and Proteobacteria. Both these studies showed that soil water availability plays an important role in structuring microbial communities. One of the reasons for the contradictory results found may be due to differences in the physical properties of the soil (Saxton & Rawls, 2006).

Soil moisture, furthermore, plays an important role in diffusion of oxygen to different areas in the soil to create aerobic conditions. However, if the soil becomes saturated with water, anaerobic conditions will develop in some areas (Grable & Siemer, 1967). These anaerobic conditions lead to a decrease in redox potential and cause anaerobic soil processes to take place such as denitrification and nitrogen fixation (Canfield et al. 2010). However, the sandy fynbos soil often allows rapid movement of water through the soil that prevents the soil from becoming anaerobic (Muofhe & Dakora, 2000).

9. Soil disturbances and effect on microbial communities

The physico-chemical properties of soil are highly influenced by soil disturbances, an event that causes a change in the functioning of an ecosystem (Griffiths & Philippot, 2013; Seybold et al. 1999). Disturbances can have different effects on microbial communities (Figure 1.3) (Allison & Martiny, 2008). In some cases, microbial communities can be resistant to a disturbance and no changes in the structure or functioning will occur. However, a disturbance can cause the community structure and function to change, but if the community is resilient, they can quickly recover and return to the original composition and function (Griffiths & Philippot, 2013). A third possibility is that microbial communities are functionally redundant i.e. where different species perform the same function. Therefore, after a disturbance the structure of the community can change, but the ecosystem function remains the same (Bender et al. 2016). Finally, if a disturbance influences microbial community structure and they are unable to adapt to these changes, a change in community structure and function will occur (Allison & Martiny, 2008).

Natural soil disturbances include events such as fires, storms, floods and insect outbreaks (Brussaard et al. 2007). Although natural disturbances can have a significant effect on soil function, the impact of anthropogenic activities is a major concern because of the enormous effect on soil function and long-term sustainability (Drenovsky et al. 2010; García-Orenes et al. 2013). One of the most destructive anthropogenic activities which disturb soil ecosystems is agricultural practices such as soil tillage, application of fertilizers and pesticides as well as monocropping systems (Drenovsky et al. 2010). In most cases, agriculture leads to soil degradation, greenhouse gas emissions, poor water quality and accumulation of pesticides (Bender et al. 2016; Bunemann et al. 2006; Mclaughlin & Mineau, 1995). Furthermore, there is a rising concern that agricultural intensification will lead to loss of soil productivity, large-scale ecosystem degradation and the loss of global biodiversity (Hartmann et al. 2015).

It is well known that microorganisms play an important role in soil quality due to their central role in virtually all soil processes (Mbuthia et al. 2015). Microbial abundance, activity and composition, therefore, play a vital role in sustainable agriculture practices (Barea, 2015; Hartmann et al. 2015). Although many studies have been done to investigate the effect of agriculture on microbial communities (Ding et al. 2013; Drenovsky et al. 2010; Figuerola et al. 2015; Lauber et al. 2013; Lauber et al. 2008), very few studies have focused on their importance in fynbos agricultural systems in the CFR. Most studies done in this region focused on specific microbial species and their role in plant health (Elliott et al. 2007; Hassen et al. 2011; Spriggs & Dakora, 2009). However, our knowledge on microbial community structure and function in fynbos agricultural soil is limited.

Two commercially important plants species indigenous to the CFR, *Aspalathus linearis* and *Cyclopia* spp., provide an ideal opportunity to compare the microbial diversity, structure and potential function in natural and disturbed ecosystems. *Aspalathus linearis* is indigenous to the north-western to western region of the Fynbos biome in the CFR and has a north to south distribution range (Joubert et al. 2008). *Cyclopia* spp. grow in the mountain slopes of the Langkloof district between the Eastern and Western Cape regions with a west to east distribution range (Figure 1.4) (Du Toit et al. 1998). *Aspalathus linearis* and *Cyclopia* spp. are both used to

prepare herbal teas respectively known as rooibos and honeybush tea. These plants have been used as folk remedies for many years by the Khoi, where they used these beverages to treat asthma, colic, headaches, nausea, digestive problems, promote lactation and cure skin rashes (McKay & Blumberg, 2007). Over the years a lot of research has been conducted to study the potential health and therapeutic applications of rooibos and honeybush teas. Both these plant species show to have strong antioxidant capacity (Inanami et al. 1995; Marnewick et al. 2003; Marnewick et al. 2005; Simon et al. 2000), chemopreventive potential (Marnewick et al. 2004; Sasaki et al. 1993), enhance the immune response (Garcia et al. 2002; Kunishiro et al. 2001; Nakano et al. 1997) and have anti-diabetic actions (Ichiki et al. 1998; Muruganandan et al. 2002). Today, rooibos and honeybush teas are mostly enjoyed as a hot beverage with added milk and sugar. Additionally, in South Africa, these teas are also enjoyed as a cold beverage, especially during the hot summer months (Joubert et al. 2008).

Prior to the twentieth century, *Aspalathus linearis* and *Cyclopia* plants were exclusively collected from the native flora. The worldwide demand for rooibos and honeybush tea is constantly increasing and this has led to a decline in plant species population in natural areas due to overharvesting (McKay and Blumberg, 2007). This has led to the establishment of commercial rooibos and honeybush farms (Joubert et al. 2008). However, establishing these plant species as crops necessitates the removal of natural fynbos and clearing of large tracts of land which may result in a loss in biodiversity (Chapin et al. 2000). How this affects soil microbial communities is still unknown. We, therefore, hypothesised that the microbial communities associated with *A. linearis* and *Cyclopia* spp. plants will be influence by agricultural activities such as soil tillage, application of pesticides as well as monocropping systems.

With the above in mind, the aim of this study was to examine the effect of commercialization of indigenous fynbos plants on this highly endemic ecosystem, with the focus on soil microbial communities. Using molecular techniques, the first objective was to characterize microbial communities associated with natural and commercially grown *Aspalathus linearis* and *Cyclopia* spp. plants. The second objective was to elucidate the possible effects these two plant species may have to

select for specific microbial communities. Finally, correlations between the microbial communities, soil properties and chemical variables were investigated over two different sampling seasons.

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Figures

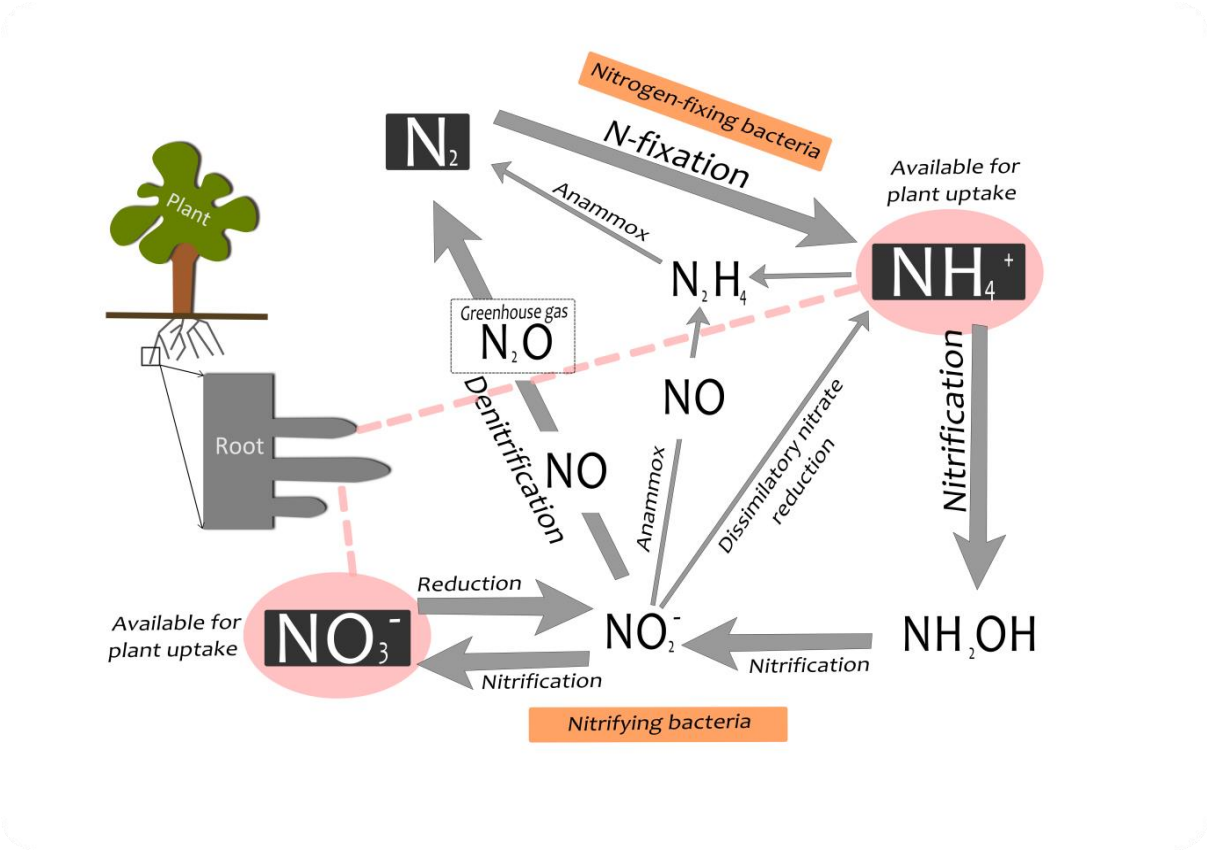

Figure1.1: Schematic diagram of the major pathways in the N cycle. Microorganisms play a critical role in reducing atmospheric N_2 to NH_4^+ through nitrogen-fixation. Under aerobic conditions, NH_4^+ can undergo nitrification to form NO_3^- or in the absence of oxygen undergo anaerobic ammonium oxidation (anammox) to release N_2 back into the atmosphere (Galloway et al. 2004; Kielland & Ganeteg, 2009).

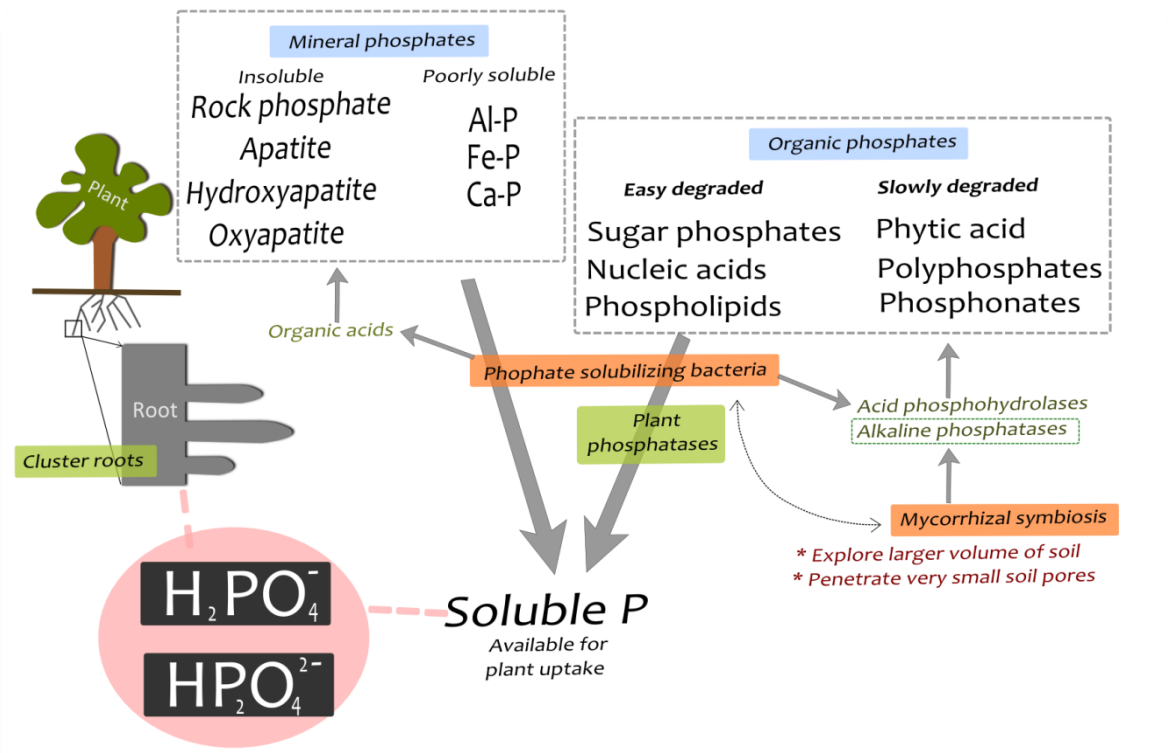

Figure 1.2: Schematic diagram of the major pathways in the P cycle. The majority of soil P is insoluble. Microorganisms are able to hydrolyse mineral and organic P through phosphatase enzymes in order to make it available for plant uptake (Maseko & Dakora, 2013; Rodríguez & Fraga, 1999).

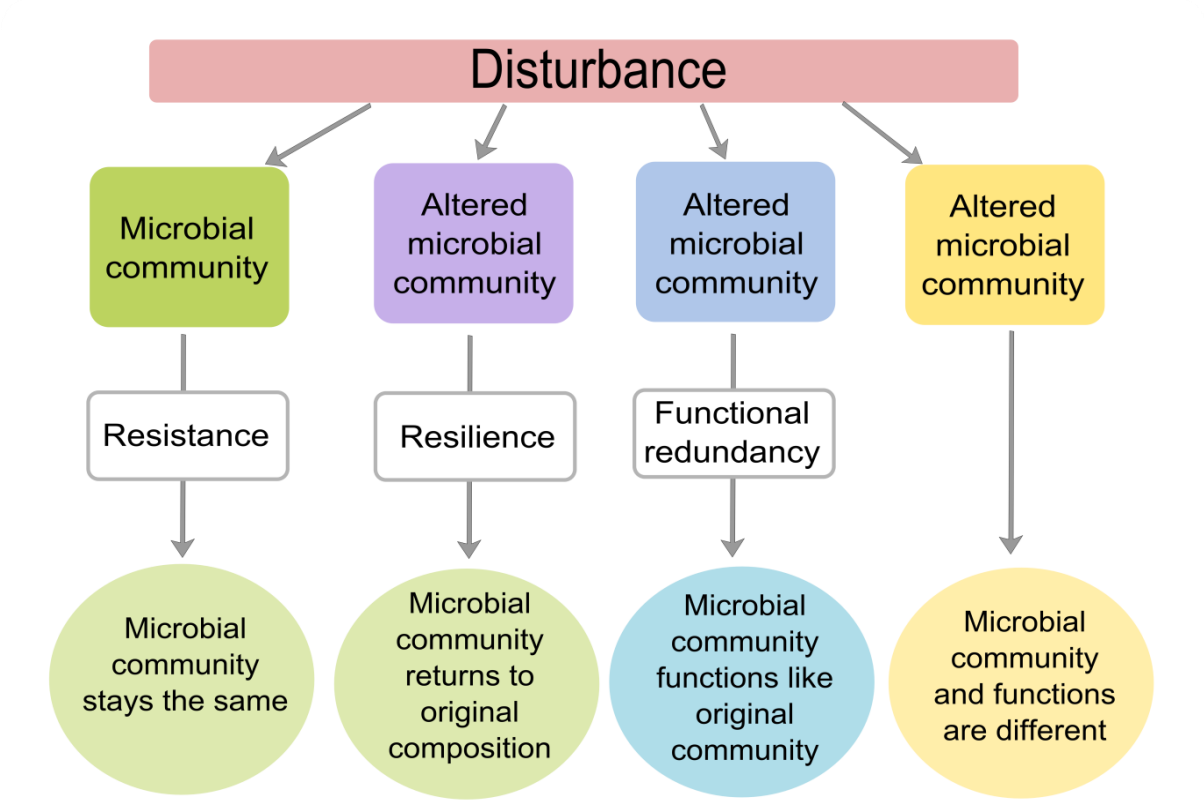

Figure1.3: Possible outcomes of a soil disturbance on the microbial community structure and function (adapted from Allison & Martiny, 2008).

Figure 1.4: Distribution map of *Aspalathus linearis* and *Cyclopia* spp. within the CFR endemic to South Africa.

Chapter 2

Soil bacterial communities associated with natural and commercial *Cyclopia* spp.

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1. Abstract

The commercially important plants in the genus *Cyclopia* spp. are indigenous to the Cape Floristic Region of South Africa and are used to manufacture an herbal tea known as honeybush tea. Growing in the low nutrient fynbos soils, these plants are highly dependent on symbiotic interactions with soil microorganisms for nutrient acquisition. The aim of this study was to investigate the soil bacterial communities associated with two commercially important *Cyclopia* species, namely *Cyclopia subternata* and *Cyclopia longifolia*. Specific interest was the differences between rhizosphere and bulk soil collected from natural sites and commercially grown plants. Samples were collected on two occasions to include a dry summer and wet winter season. Results showed that the dominant bacterial taxa associated with these plants included *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* and *Proteobacteria*. Commercial and natural as well as rhizosphere and bulk soil samples were highly similar in bacterial diversity and species richness. Significant differences were detected in bacterial community structures and co-occurrence patterns between the wet and dry seasons. The results of this study improved our knowledge on what effect commercial *Cyclopia* plantations and seasonal changes can have on soil bacterial communities within the endemic fynbos biome.

2. Introduction

The Cape Floristic Region of South Africa is recognized as one of the world's biodiversity hotspots (Cowling et al. 2003). This region has a Mediterranean climate (Cowling et al. 1996) and is characterized by many endemic plant species. *Cyclopia* spp. are part of a growing list of commercially exploited fynbos plants and occur in the coastal mountains of the Western and Eastern Cape (Du Toit et al. 1998). This woody legume is used to prepare an herbal tea known as honeybush tea (Joubert et al. 2008; Du Toit et al. 1998). The honeybush tea market is rapidly expanding, fuelled by exports to the Netherlands, Germany, and the United Kingdom. On average 200 tons of honeybush tea is produced per year, 50 tons are packed for local consumption and 150 tons for export (Joubert et al. 2011). Growing demand for honeybush tea resulted in the overharvesting of natural plants. Subsequently,

commercial producers were established in an effort to protect the ecology of this sensitive ecosystem and to keep up with demand (DAFF, 2011).

Plants are highly dependent on interactions with soil bacteria, not only to enhance plant productivity through symbiotic associations with roots (Bonkowski et al. 2000; Franche et al. 2008; Van Der Heijden et al. 2008), but also through nutrient cycling processes in the soil (Stafford et al. 2005; Van Der Heijden et al. 2008). Plants shape the microbial community structure in the rhizosphere by the release of root exudates, which contain compounds such as organic acids, phenolics, amico acids, phytosideropheres, vitamins, purines and enzymes into the soil (Dakora et al. 2002). These exudates serve as bacterial growth promoters and communication signals (Haichar et al. 2014). In return, bacteria play an important role in promoting plant growth through nutrient acquisition, nitrogen fixation, production of hormones and competition with pathogens (Dakora et al. 2002). Many biotic and abiotic factors can influence the quality and quantity of root exudates released by the plant including plant species, root age, soil temperature, water availability, and physical disturbance (Jones et al. 2004).

Studies on bacteria associated with *Cyclopia* plants mainly focused on root nodule bacteria such as *Burkholderia tuberum* (Elliott et al. 2007) and other rhizobial strains (Spriggs & Dakora 2007; Spriggs & Dakora 2009). However, these studies mainly investigated commercial plants and very little is known about the bacterial communities associated with natural *Cyclopia* spp. The need to better understand the effect of commercial agriculture on soil bacterial communities, particularly monocultured *Cyclopia* spp. in the highly endemic fynbos biome, prompted this study. In other agricultural systems factors such as agricultural practices, seasonal changes and the crop types are known to affect the structure and/or diversity of soil bacterial communities (Bell et al. 2009; Berthrong et al. 2013; Bossio et al. 1998; Montecchia et al. 2015; Smit et al. 2001). We, therefore, hypothesized that these three factors will also affect the bacterial community structure associated with *Cyclopia* fynbos agricultural systems.

The study of soil microbiomes is a challenge due the enormous diversity and complex interactions with other organisms as well as the environment (Van Der

Heijden et al. 2008). Limitations of culture-based techniques have motivated scientists to move towards molecular approaches. Next-generation sequencing (NGS) technology made significant progress over the past ten years (Metzker 2010; Mardis 2008; Van Dijk et al. 2014) and is becoming the method of choice to study microbial community structures and diversity. Using next generation sequencing we explored the soil bacterial communities associated with two commercially important *Cyclopia* species, namely *Cyclopia subternata* and *Cyclopia longifolia*. The objectives were to characterise the bacterial communities associated with rhizosphere and bulk soil of natural as well as commercially grown plants. Furthermore, we also investigated the effect of seasonal change on the bacterial communities.

3. Materials and methods

3.1 Experimental sites and sample collection

The collection of soil and plant samples was approved by the conservation authority CapeNature (permit number: 0028-AAA008-00150). Six sampling site were selected, two on each of three farms situated in the Langkloof and surrounding areas, South Africa (Table 2.1). The two sites on each farm consisted of one commercial and one natural honeybush population.

Each site was sampled in triplicate during the cold, wet winter (May 2014) and the dry, warm summer (January 2015) seasons. In total 36 bulk soil samples were collected up to a depth of 10 cm. Triplicate samples were pooled and homogenised to give a total of 12 bulk soil samples. For the collection of the rhizosphere samples, soil surrounding the plants was carefully removed to depths of 10-20 cm until roots were found. Root fragments, at least 15 cm in length, together with about 200 g of closely surrounding soil were placed in a sterile plastic bag. Rhizosphere samples of 29 plants were collected from both natural (16 plants) and commercially (13 plants) grown plants. All samples were stored on ice directly after sampling.

3.2 Abiotic soil properties

Soil samples were air dried and sieved (2 mm mesh) to remove roots and organic debris. The pH of soil slurries (1:1 1.0 M KCl solution:soil) were measured using a Crison pH-meter Basic 20+ (Crison instruments, Spain). Phosphorous (P; Bray-2P extractant) concentrations were determined with ICP-OES analysis. Total soil carbon (C) was analysed through total combustion using a Leco Truspec® CHN analyser (Seal Analytical, USA). Extractable cations (Na, K, Ca and Mg) were extracted at pH 7 with 0.2 M ammonium acetate and the concentrations were determined with ICP-OES analysis. Nitrate and ammonia were extracted from the soil with 1 M KCl. Both Nitrate-N (NO_3^--N) and Ammonium-N (NH_4^+-N) concentrations were colorimetrically determined on a SEAL AutoAnalyzer 3 (AgriLASA 2004).

3.3 DNA extraction and sequencing using Ion Torrent

DNA was extracted within 24 h of sample collection using the ZR Soil Microbe DNA kit (Zymo Research, California, USA). Following the manufacturer's protocol, 0.25 g of soil was used and 100 µl of genomic DNA was extracted from each sample. PCR amplification of the DNA was performed using primers targeting the variable V4 to V5 region of the 16S rRNA gene. The forward primers were modified with specific PGM adaptor sequences, barcodes and barcode adapters (Table S2.1) for one-way multiplex sequencing (Ion Torrent Life Technologies, Carlsbad, USA). The total reaction volume (20 µl) contained 12.5 µl of 2 x Kapa KiFi HotSart ReadyMix (Kapa Biosystems, South Africa), 0.25 µM of each primer and 1 µl DNA. PCR reactions were performed in a GeneAmp® PCR System 9700. Amplification conditions consisted of an initial denaturing step at 95 °C for 5 min followed by 35 cycles of 98 °C for 20 s, 75 °C for 15 s and 72 °C for 30 s. The reaction was completed with a final extension at 72 °C for 1 min and the samples were held at 4 °C. DNA was purified and size selected using the E-Gel® SizeSelect™ (Life Technologies, Carlsbad, USA) system. DNA concentration and size distribution of the PCR products (expected size about 400 bp) were verified using the Agilent High Sensitivity DNA Kit on the 2100 Bioanalyzer (Agilent Technologies, USA). The final concentration of each sample was adjusted to 20-25 pM and all samples were

pooled. This pooled sample was used for emulsion PCR according to the Ion PGM® Template OT2 400 Kit (Life Technologies, Carlsbad, USA). After enrichment, samples were loaded on an Ion 318™ Chip for sequencing (Ion Sequencing Kit User Guide v2.0, Life Technologies) using the Ion PGM® Sequencing 400 Kit on the PGM™ (Ion Torrent, Life Technologies).

3.4 Sequence processing and statistical analysis

Sequence data were analyzed using MOTHUR v.1.33.3 (Schloss et al. 2009; Schloss et al. 2011), following the SOP tutorial (http://www.mothur.org/wiki/Schloss_SOP) with some modifications. Ion Torrent sff. files for each barcode was generated after sequencing and assigned to the corresponding sample. The raw data files obtained from the sequencer were converted into fasta and quality files. Raw sequence data was submitted to the INSDC (EMBL-EBI/ENS, Genbank, DDBJ) with accession number DRA003953. Primers, barcodes and barcode adapters were removed from each sequence. Sequences were trimmed and filtered with a quality score greater than 25, homopolymers less than 8 and a minimum length of 400 bp. Furthermore, only the unique sequences were extracted to optimize the number of sequences. Sequences were aligned against the SILVA 115 reference database released August 23, 2013 (http://www.arb-silva.de/). Aligned sequences were screened to include sequences that started after and ended before the position that included 97.5% of the sequences. To remove columns in the alignment that did not contain any data, the alignment was filtered. Thereafter, unique sequences were extracted, followed by the pre.cluster command to remove sequences that were likely to have errors. Chimeras were removed and sequences were classified with a cutoff value of 80. To calculate uncorrected pairwise distances between aligned DNA sequences, a distance matrix was generated. The cluster.split command was then used to assign sequences to OTUs.

Alpha and β-diversity metrics are commonly used in microbial ecological studies and give valuable information with regards to the microbial community structure (Jost, 2007). The Shannon diversity and Chao1 richness indices were calculated using MOTHUR v.1.34.4 (Schloss et al. 2009) All other calculations and statistical analyses were performed in the R v3.2 software environment (R Development Core

Team 2015). Analysis of variance (ANOVA) and Tukey's honest significant differences tests were performed on the soil chemical properties, α-diversity and richness. Differences in β-diversity between the samples were tested through permutational multivariate analysis of variance (PERMANOVA, 5000 permutations) and visualised using non-metric multidimensional scaling (NMDS, Vegan Community Ecology Package V2.0-10; Oksanen et al. 2013). Significant correlations of the physico-chemical variables with the NMDS ordinations were determined using least squares linear vector fitting, after the variables were subjected to z-score standardization. The significance of the fitted vectors was determined by 1000 permutations and a Pr (>r) value < 0.05 was judged to be significant.

Interactions between bacterial taxa play an important role in shaping the community structure (Williams et al. 2014). To test co-occurrence patterns, Spearman's rank correlations were calculated between OTUs with more than five sequences. Strong correlations with an r value ≥ +0.7 and a p ≤ 0.01 were considered significant. OTU co-occurrence patterns were evaluated with the checkerboard score (C-score) to confirm that the co-occurrence patterns were non-random (Stone & Roberts 1990). Networks were created with the igraph package and subsequently visualized with the open source platform Cytoscape 3.2.1, using the Fruchterman-Reingold layout (Shannon et al. 2003).

4. Results

4.1 Bacterial community composition

After quality filtering, a total of 88523 non-chimeric unique bacterial sequences were obtained (Table S2.2). Operational taxonomic units (OTUs) were classified to 38 orders. Only 10 of these orders occurred with a mean relative abundance of ≥1% and belonged to four phyla, namely *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* and *Proteobacteria*. The majority of the *Actinobacteria* sequences were classified to the *Actinomycetales* (91%). The *Burkholderiales* (32%) and *Rhizobiales* (32%) were the most abundant orders within the *Proteobacteria*. *Acidobacteria* and *Bacteroidetes* were mainly represented by the *Acidobacteriales* and *Sphingobacteriales*, respectively (Figure S2.1).

All the soil samples were dominated by the order *Actinomycetales* with a mean relative abundance of 49 ± 17.25 %, followed by *Acidobacteriales* (16 ± 11 %) (Figure 2.1A). The overall structure of taxa occurring in commercial and natural (Figure 2.1C), as well as bulk and rhizosphere (Figure 2.1D) soil samples were similar. However, the relative abundance of the *Burkholderiales* was higher in the rhizosphere (9 ± 4.3 %) compared to bulk (2 ± 2 %) soil samples. Significant differences in taxa composition was observed between soil sampled in the wet and dry seasons. Notably, the *Sphingomonadales* was mainly detected in soil samples collected in the wet season, whereas the *Burkholderiales*, *Solirubrobacterales*, *Sphingobacteriales* and *Xanthomonadales* were mostly detected in soil samples collected in the dry season (Figure 2.1B).

4.2 Bacterial OTU diversity and species richness

The Shannon diversity and Chao 1 total species richness indices were not significantly different between samples collected from commercial and natural sites, bulk and rhizosphere soil fractions or the different farms ($p>0.05$). However, samples collected in the dry season had a significantly higher bacterial diversity ($F=62.29$ and $p<0.001$) and species richness ($F=12.67$ and $p<0.001$) compared to the wet season (Figure 2.2).

Similarly, there was a significant (PERMANOVA $p<0.05$) difference in β-diversity between the wet and dry seasons (Figure 2.3). Significant fitted vectors (Table S2.3) showed that the carbon and soil resistance levels were higher during the wet than the dry season. No statistically significant difference could be observed in beta-diversity between any of the farms. However, some samples from Guava Juice and Heights had increased NO_3, P, K, Mg, Ca and pH levels. Whereas, increased Na and H^+ were observed in samples from the farm Montagri.

4.3 Network analysis

Significant differences were detected in bacterial community composition and diversity between samples collected in the wet and dry seasons. Due to these differences, we constructed co-occurrence patterns of possible interactions that

could occur between bacterial taxa present in the soil collected during these two seasons. Clear differences were seen in the non-random co-occurrence patterns of bacterial communities present in the wet (Figure 2.4) and dry seasons (Figure 2.5).

The soil microbial co-occurrence network of the wet season samples composed of 178 nodes and 386 edges (Figure 2.4A). This network was characterised by strong co-occurrence patterns between the *Acidobacteriaceae* and families of bacteria belonging to the *Actinobacteria* namely *Acidothermaceae*, *Geodermatophilaceae*, *Micromonosporaceae*, *Mycobacteriaceae* and *Pseudonocardiaceae*. The environmental variables also showed strong correlations with these families of bacteria. Furthermore, the mean degree (number of correlations) of each family tends to be independent of its mean relative abundance over all samples collected during the wet season (Figure 2.4B).

The microbial network for the dry season soil samples consisted of 146 nodes and 537 edges (Figure 2.5A). This network showed fewer interactions between bacterial OTUs from different families. The OTUs of the *Acidothermaceae* and other families from *Actinobacteria* tend to co-occurred, forming small clusters of interconnections. Prominent interconnections were also observed between the *Acidothermaceae*, *Burkholderiaceae* and *Bradyrhizobiaceae*. In the dry season strong correlations were observed between environmental variables. However, fewer interactions were observed between environmental variables and bacterial OTUs, which included the *Acidobacteriaceae*, *Acidothermaceae* and other *Actinobacteria*. In contrast to the wet season, the mean degree of the nodes correlated with the mean relative abundance of the families (Figure 2.5B). The *Acidothermaceae* was the most abundant and had the greatest interconnectivity (mean degree %). All the families present in the dry season network further showed a lower mean relative abundance and degree percentages compared to the wet season. An exception is the *Solirubrobacteriaceae* which had a high degree percentage although it had the lowest mean relative abundance. This may be due to their co-occurrence with the *Acidothermaceae*.

5. Discussion

Over the past few years, NGS techniques were extensively used to get a better understanding of the complex interactions and functions of microbial communities (Fierer et al. 2007; Fujimoto et al. 2014; Ju & Zhang 2014; Prober et al. 2015; Rampelotto et al. 2015; Taketani et al. 2015). Using the Ion Torrent PGM sequence data, we investigated bacterial communities associated with commercially important *Cyclopia* spp., focussing on taxonomic composition, relative abundance, diversity and co-occurrence. We hypothesized that agricultural practices, seasonal changes and the rhizosphere of *Cyclopia* plants will have an effect on the structure and/or diversity of soil bacterial communities.

In this study we showed that *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* and *Proteobacteria* were the most dominant bacterial groups in the low nutrient fynbos soil. This is in agreement with other studies on soil bacterial communities in arid and semi-arid regions characterised by long periods of dry warm summers (Acosta-Martínez et al. 2014; Kavamura et al. 2013; Taketani et al. 2015), and Mediterranean climates (Bachar et al. 2010).

Bacterial communities in the rhizosphere are known to be less diverse and greater in abundance compared to bulk soil (Dennis et al. 2010; Smalla et al. 2001; Uroz et al. 2010). In contrasted to this observation, we found no statistically significant difference between the bulk and rhizosphere soil fractions (α-diversity, β-diversity and species richness). This may be explained by the farming practices and/or the physiology of *Cyclopia* spp. These factors could also account for the similarity observed between natural and commercial samples. Farmers plant *Cyclopia* plantations in semi-natural areas already populated with other fynbos plants. This results in very dense plant growth in commercial fields, comparable to that of plants occurring in natural areas (Figure S2.2). It is also well known that plants release root exudates which are plant specific and serve as signals to select for particular bacterial communities (Bais et al. 2001; Bais et al. 2006; Dennis et al. 2010; Maseko & Dakora 2013). The root-systems of *Cyclopia* plants are deep-rooted with very long lateral roots that may affect soil microbes in large areas surrounding the plant (Spriggs & Dakora 2009). Due to the dense plant growth, long lateral roots and

specific root exudates released by *Cyclopia* plants, bulk soil samples were most likely affected by root exudates of *Cyclopia* and other fynbos plants in the area. Furthermore, the effect on the soil microbial communities by the root exudates, appear to be the same in natural and commercial areas. Adding to this, no significant differences in the chemical composition between bulk and rhizosphere soil fractions as well as natural and commercial areas were detected, and may explain why no differences in bacterial communities were observed.

Despite the similarity in diversity and species richness between the bulk and rhizosphere soil fractions, a significant difference was detected in the relative abundance of the *Burkholderiales*. This group was more abundant in the rhizosphere soil samples (Fig. 1D). In the CFR, the most dominant root nodule forming bacteria associated with legumes, including *Cyclopia* spp., are *Burkholderia* species (Beukes et al. 2013; Elliott et al. 2007; Lemaire et al. 2015; Spriggs & Dakora 2009). Root nodule bacteria fix atmospheric dinitrogen making it available for the plant, in exchange for organic carbon compounds (Lionel et al. 2001). This mutualistic interaction may be critical for both plants and bacteria, especially in the nutrient limited fynbos soils.

The factor which appeared to have the greatest effect on the microbial communities was the sampling season. Evidence was presented in this study that supports the hypothesis that seasonal changes affect the bacterial communities in fynbos soil (Bell et al. 2009; Smit et al. 2001). Differences in soil temperature may be a major reason for the observed change in bacterial community structure between the cold wet and the warm dry seasons. It is well known that temperature directly affects microbial activity and community structure in soil (Pietikäinen et al. 2005). Furthermore, root exudation by plants is likely to change between seasonal growth cycles and developmental phases (Aulakh et al. 2001) which can affect the bacterial communities.

The significant grouping of samples during the two different seasons (Figure 2.3) could also be due to changes in soil properties (Stevenson et al. 2014). During wet seasons, plant material is degraded more rapidly than in dry seasons. Higher abundance of organic matter is thus present (Denef et al. 2001) and, as also seen in

this study, will lead to an increase of carbon levels in the soil. Lower diversity and species richness was observed in the wet season. When conditions become more favourable, some microorganisms able to quickly utilize the increased C-sources, can proliferate faster than other slow-growing taxa. Usually, this leads to a less diverse community structure due to the dominance of certain groups of fast-growing taxa (Van Gestel et al. 1993). This may be the case for members of the metabolically versatile *Sphingomonadales* which were more abundant in soil samples collected during the wet season (Figure 2.1B). This also supports the findings of Bachar and co-workers (2010), where an increase of *Sphingomonadaceae* was detected along higher precipitation gradients within Mediterranean, semi-arid and arid climatic regions. In the dry season *Burkholderiales*, *Solirubrobacterales*, *Sphingobacteriales* and *Xanthomonadales* were found to be more abundant, similar to other dry and nutrient poor soils (Chong et al. 2011; Rampelotto et al. 2015). This higher diversity and species richness observed in the dry season might be beneficial for functional redundancy (Taketani et al. 2015).

The differences in alpha and β-diversity between the seasons are usually influenced by environmental variables as well as the relationship between microbial taxa (Barberán et al. 2012; Ju & Zhang 2014). Therefore, we also generated co-occurrence patterns between taxa, comparing samples from the wet and dry seasons. Co-occurrence in these networks are most likely due to bacterial taxa sharing similar ecological niches and not due to symbiotic interactions (Allison & Martiny 2008; Barberán et al. 2012; Bell et al. 2009; Faust & Raes 2012).

As anticipated, correlation networks from the wet (Figure 2.4) and dry (Figure 2.5) seasons showed significant differences in co-occurrence patterns and taxa involved. We considered two possible explanations for these differences. First, the high diversity of interconnected bacterial OTUs and environmental variables present in co-occurrence patterns of the wet season might be due to an increase of water availability and nutrients in the soil. In wet environments bacteria and nutrients are able to be transported more easily via water which is otherwise constricted in dry environments (Abu-ashour et al.1994). The movement of bacteria and nutrients in the soil, therefore, increase the likelihood of possible interactions between different

taxa, as detected in this study. Small clusters of interconnected OTUs from families of the *Actinobacteria* dominated the interactions in the dry season and might also be an indication of the ability of this group to tolerate drought conditions. A second possibility explaining changes in community networks may be due to a change in root exudate composition. It has been reported that root exudates can respond rapidly to changes in the environment. It is therefore, likely that seasonal changes can affect the root exudates released by *Cyclopia* spp. and subsequently alter the microbial community structures (Dilkes et al. 2004; Rovira 1969).

6. Conclusion

To our knowledge, this is the first report that characterizes total soil bacterial community structures associated with the commercially important fynbos plants, *C. subternata* and *C. longifolia*. This study provides evidence that bacterial communities are highly similar in soil collected from natural and commercially grown plants. Therefore, the current practice of planting *Cyclopia* in commercial plantations appears to have little effect on the soil bacterial communities. Significant changes in community structures and co-occurrence patterns between the two sampling seasons support the contention that microbial taxa adapt and resist environmental changes differently. In order to fully understand and evaluate the effect of seasonal changes on bacterial communities associated with *Cyclopia* spp., future research should sample more frequent over a longer time period. Evaluating changes in soil moisture levels and root exudates in rhizosphere soil over time, may also give a better understanding to why these changes occur.

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Figures and Tables

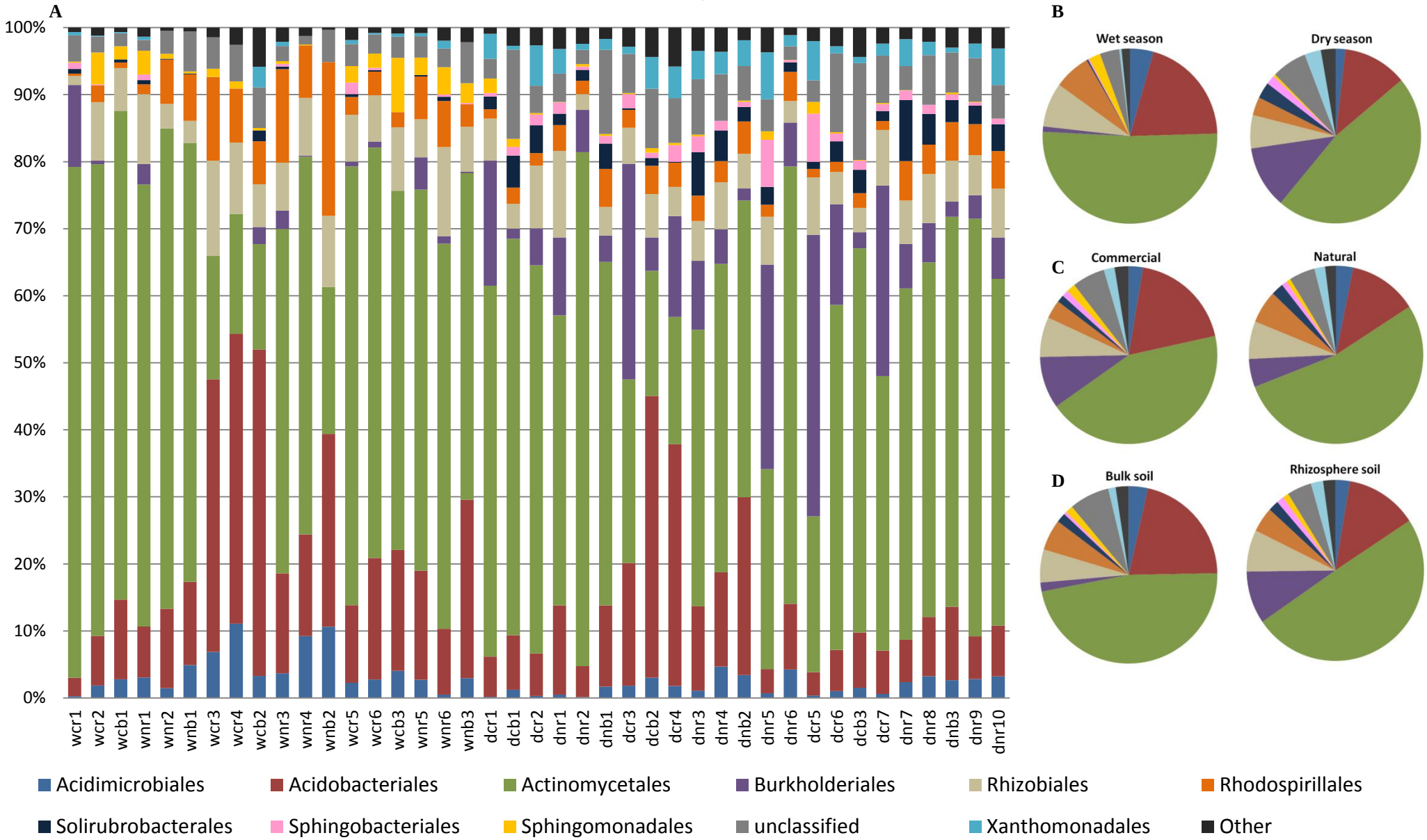

Figure 2.1: Relative abundance of classified sequences at order level within all samples (see Table S2.2 for sample description). Sequences with a homology ≥ 80% were used and only orders with a mean relative abundance of ≥ 1% were included. A.) Distribution of orders in all samples. B.) Samples grouped based on sampling time (wet or dry season), C.) sample type (commercial or natural sites), and D.) soil fraction (bulk or rhizosphere soil).

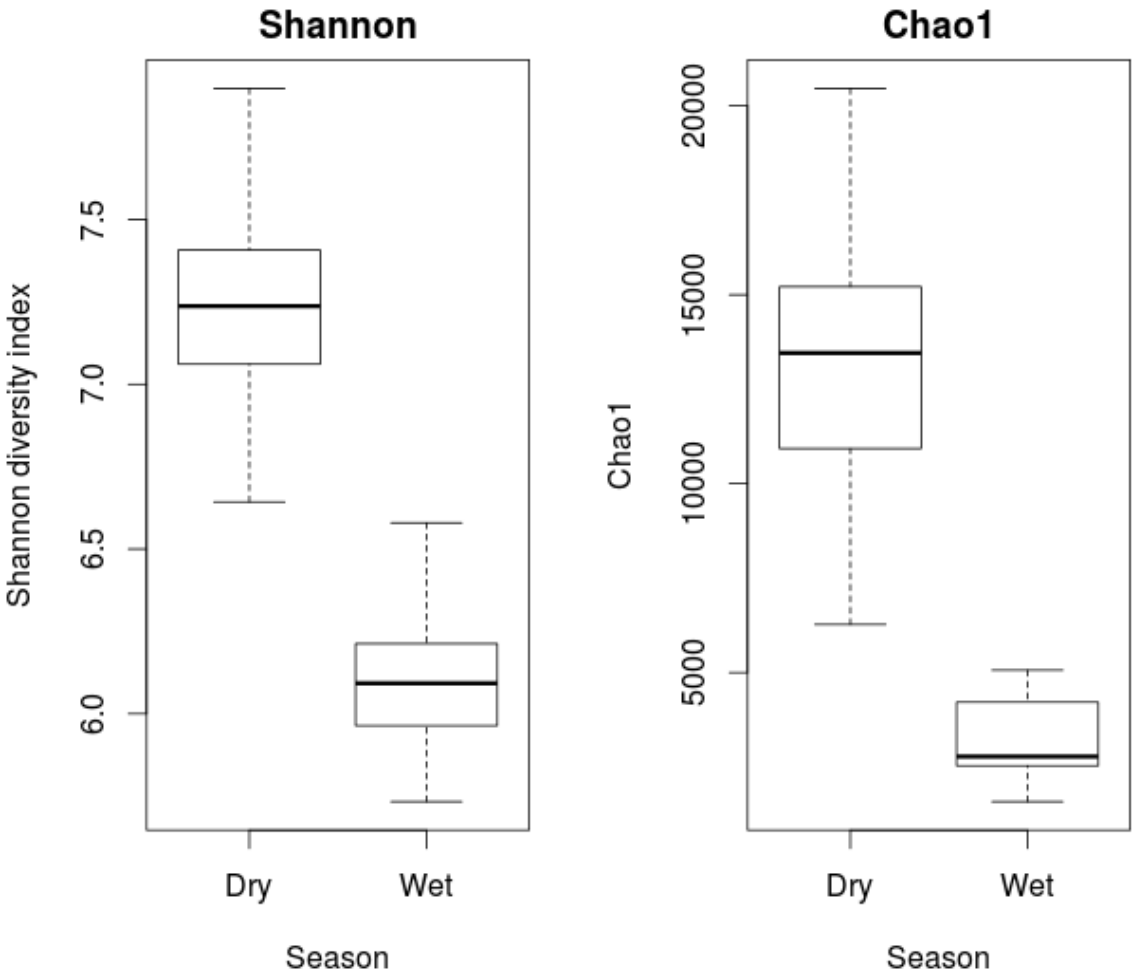

Figure 2.2: Shannon's diversity index (ANOVA significance values of $p<0.001$ and $F=62.29$) and total species richness obtained by the Chao 1 index (ANOVA significant difference values of $p<0.001$ and $F=12.67$) for bacterial communities between the different sampling times.

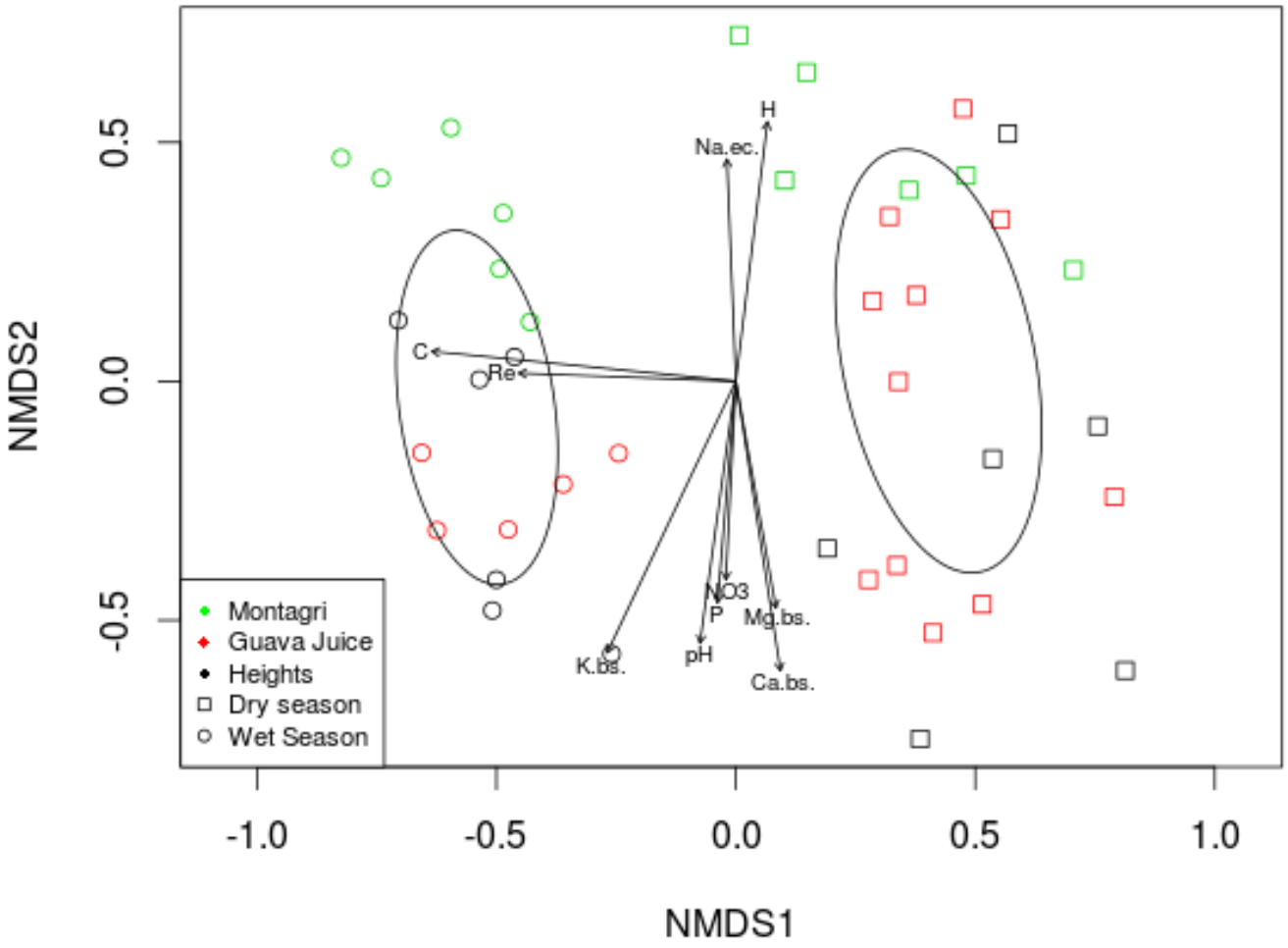

Figure 2.3: Non-metric multi-dimentional scaling ordination plot of bacterial communities based on the Bray-Curtis distance. Ellipses represent the samples which were within 95% confidence limit and included the wet and dry season (Stress=0.185). A bi-plot is overlaid on the ordination to display soil chemical variables that have a significant correlation ($p<0.05$) with the microbial community structure (ec: exchangeable cations; bs: basis saturation).

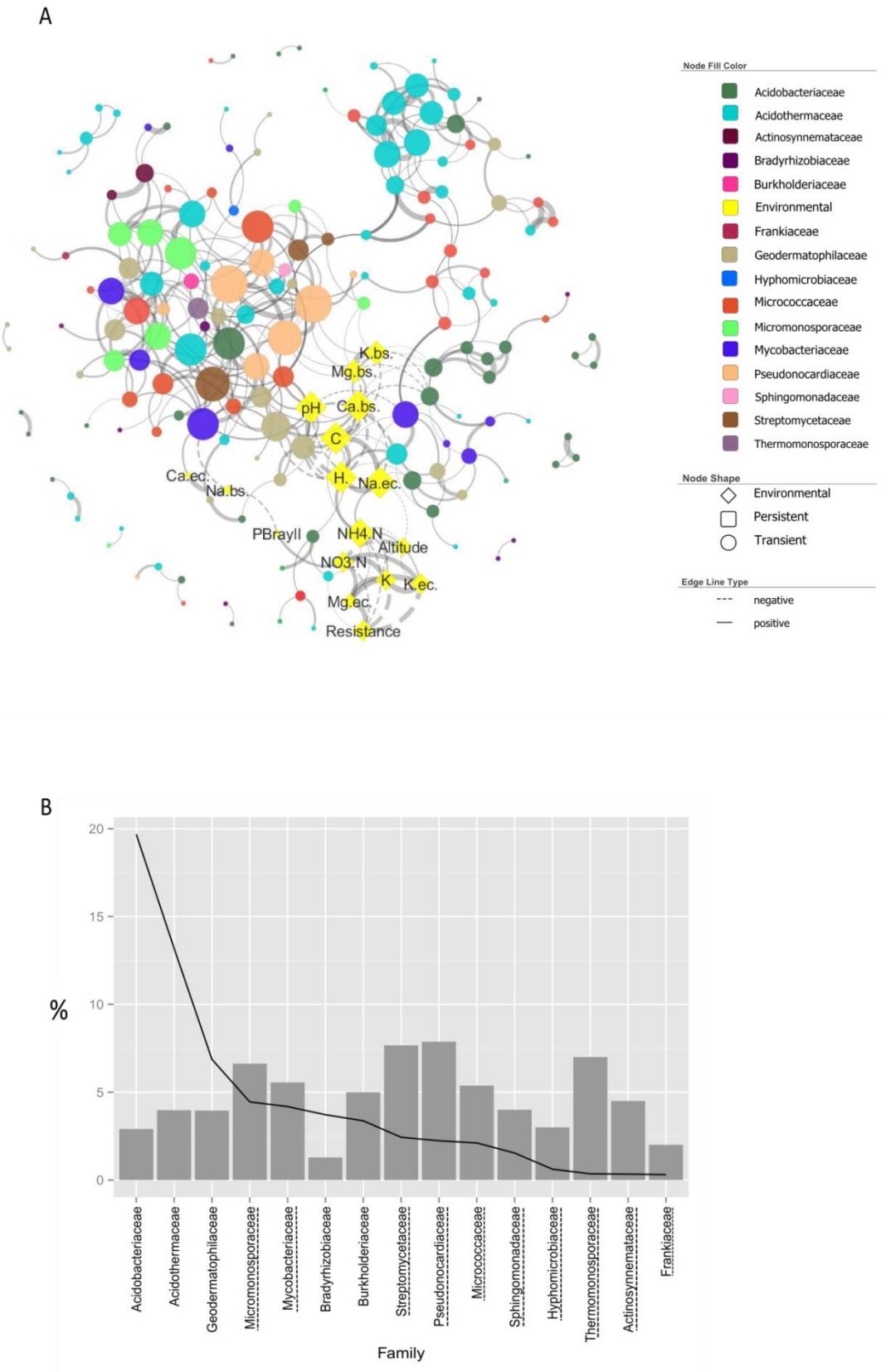

Figure 2.4: A.) Taxonomic-environmental network created from strong and significant spearman correlations ($r>0.7$ and $p\leq0.01$). Network indicates relationships between environmental variables and bacterial groups co-occurring in the wet season. Node size of each OTU is proportional to the number of connections and the line thickness proportional to the absolute value of local similarity. Edge line type indicates a positive or negative neighbour interaction. The network composed of 178 nodes and 386 edges (ec: exchangeable cations; bs: basis saturation). B.) Mean relative abundance (%) of microbial taxonomic groups, classified to family level, present in the wet season (black line). Degree (%) of taxonomic groups present in co-occurrence patterns are represented by the grey bars. Underlined family names indicate that the family is not present in co-occurrence patterns in the wet season (Figure 2.5)

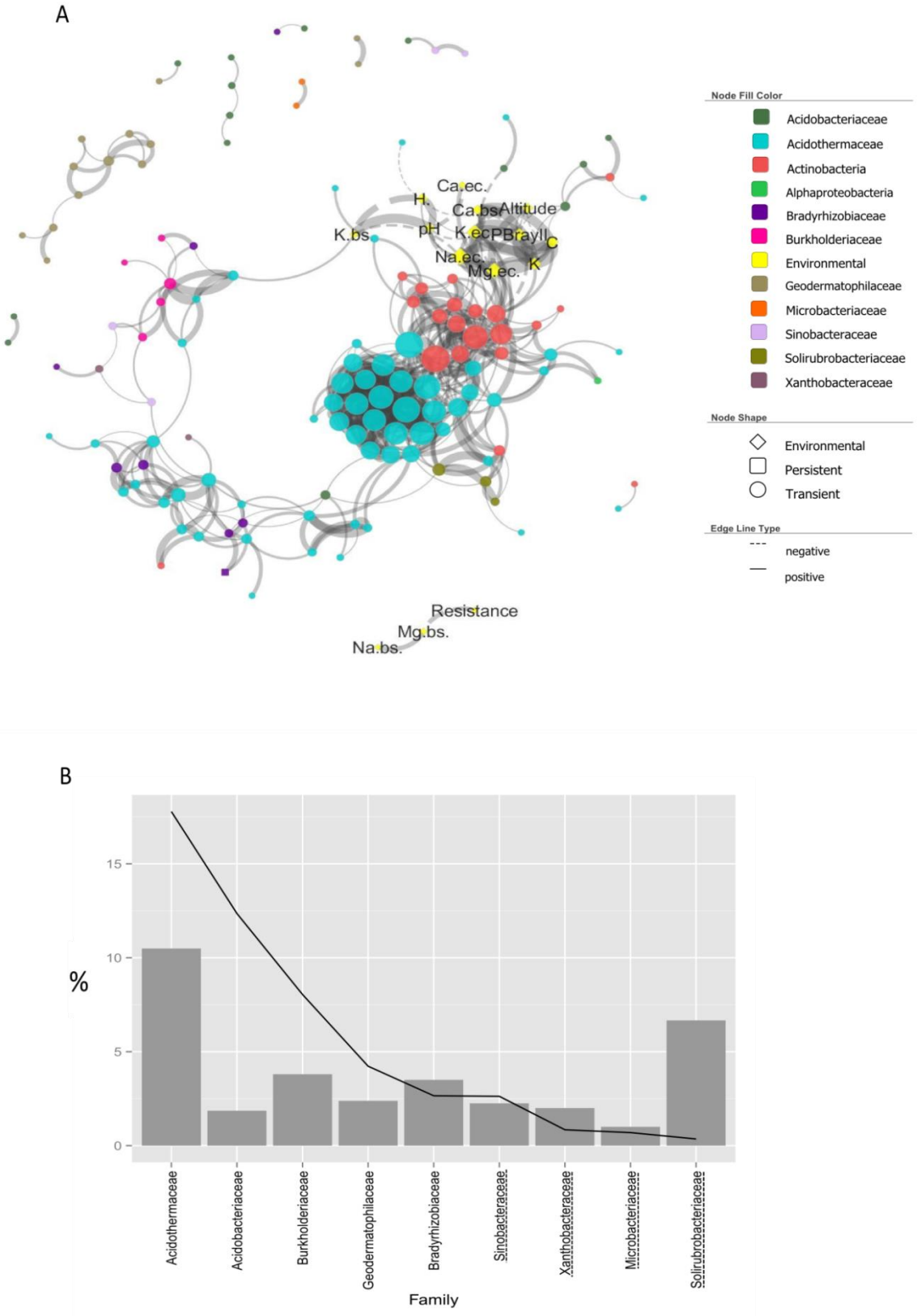

Figure 2.5: A.) Taxonomic-environmental network created from strong and significant spearman correlations (r>0.7 and p≤0.01). Network indicates relationships between environmental variables and bacterial groups co-occurring in the dry season. Node size of each OTU is proportional to the number of connections and the line thickness proportional to the absolute value of local similarity. Edge line type indicates a positive or negative neighbour interaction. The network composed of 146 nodes and 537 edges (ec: exchangeable cations; bs: basis saturation). B.) Mean relative abundance (%) of microbial taxonomic groups, classified to family level, present in the dry season (black line). Degree (%) of taxonomic groups present in co-occurrence patterns are represented by the grey bars. Underlined family names indicate that the family is not present in co-occurrence patterns in the wet season (Figure 2.4).

Table 2.1: Summary of sampling sites locations, type of soil, season and sample numbers

Sample number	Soil fraction	GPS coordinates		Type	Altitude	Season	Farm
wcr1, wcr2	Rhizosphere	S34°01.797'	E24°20,189'	Commercial	227	Wet	Guavajuice
wcb1	Bulk soil						
wnr1, wnr2	Rhizosphere	S34°02.802'	E24°20.812'	Natural	181		
wnb1	Bulk soil						
dcr5, dcr6, dcr7	Rhizosphere	S34°01,851'	E24°20,187'	Commercial	222	Dry	
dcb3	Bulk soil						
dnr7, dnr8, dnr9, dnr10	Rhizosphere	S34°02,851'	E24°20,795'	Natural	167		
dnb3	Bulk soil						
wcr3, wcr4	Rhizosphere	S33°58.975'	E24°12.989'	Commercial	538	Wet	Montagri
wcb2	Bulk soil						
wnr3, wnr4	Rhizosphere	S33°59.156'	E24°12.390'	Natural	473		
wnb2	Bulk soil						
dcr3, dcr4	Rhizosphere	S33°59,075'	E24°13,100'	Commercial	495	Dry	
dcb2	Bulk soil						
dnr3, dnr4, dnr5, dnr6	Rhizosphere	S33°58,998'	E24°12,258'	Natural	473		
dnb2	Bulk soil						
wcr5, wcr6	Rhizosphere	S33°52.131'	E23°59.102'	Commercial	557	Wet	Heights
wcb3	Bulk soil						
wnr5, wnr6	Rhizosphere	S33°51.545'	E23°58.316'	Natural	561		
wnb3	Bulk soil						
dcr1, dcr2	Rhizosphere	S33°52,139'	E23°59,157'	Commercial	563	Dry	
dcb1	Bulk soil						
dnr1, dnr2	Rhizosphere	S33°51,847'	E23°58,305'	Natural	568		
dnb1	Bulk soil						

Chapter 3

Bacterial communities associated with natural and commercially grown rooibos (*Aspalathus linearis*)

This chapter was submitted to Microbial Ecology – currently under review

1. Abstract

Aspalathus linearis is a commercially important plant species endemic to the Cape Floristic Region of South Africa and is used to produce an herbal tea known as rooibos. Symbiotic interactions between *A. linearis* and soil bacteria play an important role in the survival of these plants in the highly acidic, nutrient poor fynbos soil. The aim of this study was to characterize and compare rhizosphere and bulk soil bacterial communities associated with natural and commercially grown *A. linearis* plants. The study extended over two sampling seasons to investigate the possible effect seasonal change may have on these communities. Bacterial communities were characterized with high throughput sequencing and were correlated with chemical soil properties. Actinobacteria, Proteobacteria and Acidobacteria were the most dominant bacterial phyla detected in this study. Highly similar bacterial communities were associated with natural and commercially grown plants. However, significant differences in diversity and structure were observed between samples collected during the different seasons. Rhizosphere and bulk soil samples collected in the dry season were also significantly different. This study gives a better insight into the structure and some of the factors that can shape bacterial communities associated with the commercially important *A. linearis*.

2. Introduction

The Cape Floristic Region (CFR) in South Africa has an exceptionally high floral diversity and is recognized as one of the world's biodiversity hotspots (Cowling et al. 2003). This Mediterranean climate region is known for warm, dry summers and cool, wet winters with nutrient-poor, acidic soils (Cowling et al. 1996; Cowling et al. 2003; Richards et al. 1997; Van Wilgen et al. 2012). Endemic to the CFR is the commercially important plant, *Aspalathus linearis* (Burm. f.) R. Dahlgren. *Aspalathus* is the second largest genus of vascular plants in this region, with as many as 279 known species (Malgas et al. 2010). This shrub-like bush belongs to the family Leguminosae and has a geographical distribution range from the north-western to western region of the CFR (Hawkins et al. 2011).

The needle-like leaves and stems of *A. linearis* are used to make an herbal tea called Rooibos tea, which is well known for its numerous health properties (Joubert et al. 2008; Marnewick et al. 2011). Rooibos refers to the red colour of the leaves, which is a result of the fermentation step of the green plant material during production of the tea (McKay & Blumberg, 2007). The rooibos industry is well established in South Africa with a large local and international market. In 2014, South Africa produced approximately 12 500 tons of rooibos tea, of which 4500 - 5000 tons were destined for local markets. The rest was exported to Germany, Japan, Netherlands, United Kingdom, and the United States of America (DAFF, 2014).

The continued increase in demand for rooibos tea has led to the establishment of many successful farms. However, cultivation of *A. linearis* remains challenging due to the highly endemic nature of the CFR. Plants in this region, including *A. linearis*, are well adapted to survive these harsh conditions (Lambers et al. 2011). For example, *A. linearis* is able to maximize nutrient acquisition by increasing the surface area of the roots with specialized structures known as root clusters (Skene, 1998). Furthermore, these plants release specific root exudates (OH^- and HCO_3^-) that can modify the pH of the rhizosphere soil. A less acidic rhizosphere environment enhances nutrient uptake and creates a more favorable environment for symbiotic interactions to take place (Dakora & Phillips, 2002). In addition, root exudates serve as communication signals and nutrients for soil bacteria (Bais et al. 2004). Symbiotic interactions between plants and soil bacteria may play a critical role in the survival of *A. linearis*. Moreover, bacteria are essential in soil nutrient cycling processes and also enhance plant productivity through nitrogen fixation, nutrient acquisition and the production of various growth factors (Cloete et al. 2007; Doornbos et al. 2011; Maseko & Dakora, 2013; Masson-Boivin et al. 2009).

Previous studies which investigated the interactions between soil microbiota and *A. linearis* mostly focused on root nodulating bacteria, collectively referred to as rhizobia (Dakora, 2012; Hassen et al. 2011; Muofhe & Dakora 2000). Under the nitrogen-limiting conditions of CFR soil, these bacteria are able to induce the formation of specialized plant structures known as root nodules. Within the root nodule bacteria fix atmospheric nitrogen, making it available for the plant (Doornbos

et al. 2011; Masson-Boivin et al. 2009). Rhizobia species isolated from *A. linearis* include members of the α-Proteobacteria (*Mesorhizobium* and *Rhizobium*) as well as β-Proteobacteria (*Burkholderia* and *Herbaspirillum*) (Hassen et al. 2011). Although rhizobial species are functionally very important, this group of bacteria represents only a small fraction of the vast diversity of soil bacteria with which *A. linearis* could come in contact with (Dance, 2008).

Very little is known about the diversity and structure of bacterial communities associated with *A. linearis*. Also, it is unclear what effect agricultural practices have on soil bacterial communities. Commercial and natural *Aspalathus linearis* populations therefore, provide an ideal opportunity to compare the bacterial communities of natural and disturbed CFR soil. Bacteria play a central role in the functioning of soil ecosystems and complex interactions constantly occur between bacteria, plants and the environment (Cotner & Biddanda, 2002; Falkowski et al. 2008). Many factors, such as seasonal changes, geographic location and agricultural practices (Barea, 2015; Stevenson et al. 2014, Tsiknia et al. 2014) can influence these complex interactions and alter bacterial community structure and function (Allison & Martiny, 2008). We hypothesized that these factors will also affect the bacterial communities associated with *A. linearis*.

Due to the enormous complexity of soil microbial communities, molecular biology techniques such as next generation sequencing are often used to investigate them (Kirk et al. 2004; Metzker, 2010; Postma et al. 2016; Van Dijk et al. 2014). The aims of this study were to characterize and compare soil bacterial communities associated with natural and commercially grown *A. linearis* plants. In addition, we also investigated the effect seasonal change may have on the bacterial communities.

3. Materials and methods

3.1 Site description and sample collection

A permit was obtained from the conservation authority CapeNature (permit number: 0028-AAA008-00150) in order to sample soil and plant material. Samples were collected from two farms, Klipopmekaar and Kleinvlei, located in the Western Cape

Province, South Africa (Table 3.1). Each farm consisted of natural and commercial sampling sites. Natural sites were characterized by dense fynbos vegetation. Conversely, rooibos plants in commercial plantations were easily accessible because there were no other fynbos plants nearby (Figure S1).

Samples were collected in January 2014 (warm, dry summer) and September 2014, (cold, wet winter). During the collection of rhizosphere samples, soil surrounding the plants was removed up to depths of 10 to 20 cm in order to expose roots. Root fragments with a minimal length of 15 cm were collected together with approximately 200 g of surrounding soil. In total 25 plants (12 commercial and 13 natural) were sampled. At the same time, bulk soil samples were collected for each GPS location where plants were sampled. Each bulk soil sample consisted of triplicate samples, which were pooled and homogenized to give a total of 17 samples (12 commercial and 5 natural). All samples were placed in sterile plastic bags and stored on ice directly after sampling.

3.2 Abiotic soil properties

Abiotic soil properties were measured for each soil sample as described by Postma *et al*. 2016 (Chapter 2). Soil properties measured included soil resistance, pH, phosphorous, total soil carbon, extractable cations (Na^+, K^+, Ca^{2+} and Mg^{2+}), nitrate and ammonia.

3.3 DNA extraction, Ion Torrent sequencing and data processing

Total genomic DNA was extracted from 0.25 g of soil for each sample within 24 hours after sampling using the ZR Soil Microbe DNA kit (Zymo Research, California, USA). After confirming the presence of DNA on a 1 % agarose gel, PCR amplifications were performed. The primer set that was used targeted the variable V4 to V5 region of the 16S rRNA gene (~400 base pairs). For one-way multiplex sequencing on the Ion Torrent (Life Technologies Carlsbad, USA), all forward primers were modified as described by Postma et al. 2016 (Table S3.1). DNA libraries were constructed in 20 µl PCR reaction mixtures which contained 12.5 µl of 2 x Kapa KiFi HotSart ReadyMix (Kapa Biosystems, South Africa), 0.25 µM of each

primer and 1 µl total genomic DNA. Amplification conditions consisted of an initial denaturing step at 95 °C for 5 min followed by 35 cycles of 98 °C for 20 s, 75 °C for 15 s and 72 °C for 30 s. The reaction was completed with a final extension at 72 °C for 1 min and the samples were held at 4 °C. PCR reactions were performed in a GeneAmp® PCR System 9700.

Amplified DNA was prepared for Ion Torrent sequencing as described by Postma et al. 2016 (Chapter 2). After purification, size selection and PCR enrichment, samples were template using the Ion OneTouch™ 2 System and loaded onto an Ion 318™ Chip for sequencing (Ion Sequencing Kit User Guide v2.0, Life Technologies) using the Ion PGM® Sequencing 400 Kit on the PGM™ (Ion Torrent, Life Technologies). Raw data files (.sff) for each barcode was assigned to the correct sample and sequence data was submitted to the INSDC (EMBL-EBI/ENS, Genbank, DDBJ) with accession number DRA004000. Sequence quality control and processing was done as described by Postma et al. 2016 (Chapter 2) using MOTHUR v.1.33.3 (Schloss et al. 2009; Schloss et al. 2011).

3.4 Statistical analysis

The Shannon diversity and Chao1 species richness (Hill, 1973; Hill et al. 2003) were calculated using MOTHUR v.1.33.3. All other calculations and statistical analyses were performed using the R v3.2 software environment (R Development Core Team 2015). Significant differences between the α-diversity of different samples were calculated using analysis of variance (ANOVA) and Tukey's honestly significant difference post hoc test. Permutational multivariate analysis of variance (PERMANOVA, 5000 permutations) was used to test differences in β-diversity between the samples. Results were visualized using non-metric multidimensional scaling (NMDS, Vegan Community Ecology Package V2.0-10; Oksanen et al. 2013). Significant correlations of the soil chemical variables with the NMDS ordinations were determined using least squares linear vector fitting, after the variables were subjected to z-score standardization. Significance of the fitted vectors was determined by 1000 permutations and a Pr (>r) value <0.05 was judged to be significant.

In order to get a more comprehensive understanding of the roles specific bacterial taxa may play in communities (Lynch & Neufeld, 2015), indicator OTU analysis were performed using different seasons and rhizosphere soil fractions as categorical variables. Indicator OTUs were the classified up to order or family level and relative abundance of indicators were determined for each sample. Analysis was done using MOTHUR v.1.33.3 (Dufrene & Legendre, 1997).

Interactions between bacteria and their physico-chemical environment play an important role in shaping the communities (Bloor & Bardgett, 2012; Drenovsky et al. 2010). Therefore, Spearman's rank correlations between OTUs and soil chemical variables were calculated. Only bacterial taxa that showed significant correlations ($p < 0.05$ and $r \geq 0.5$) were used to construct a correlogram using the corrgram package in R (Tsiknia et al. 2014).

4. Results

4.1 Bacterial OTU diversity and species richness

No significant differences in the Shannon diversity index and Chao 1 species richness ($p>0.05$) were detected between samples from the different farms, season or type of soil. However, a significantly ($p<0.05$) higher Shannon diversity index was calculated for the bulk soil samples collected in the dry season compared to the rhizosphere samples from both dry and wet seasons (Figure 3.1). The diversity in bulk soil samples collected in the wet season showed no significant difference to any of the rhizosphere or bulk soil samples.

A significant difference (PERMANOVA $p<0.05$) in β-diversity was detected between samples collected in the dry and wet seasons (Figure 3.2). Furthermore, the bulk and rhizosphere soil samples collected during the dry season grouped separately. However, this separate grouping was not observed in samples from the wet season. Statistically significant fitted vectors (Table S3.2) showed that K^+ and Na^+ concentrations were higher in rhizosphere soil samples collected during the dry season compared to the other samples. Both these ions play important roles in plant nutrition and the homeostasis of bacterial cells (Corratgé-Faillie et al. 2010).

Samples collected during the wet season had higher C concentrations, C/N ratios and soil resistance compared to the dry season samples.

4.2 Bacterial community composition and indication taxa

A total of 189,133 high quality, non-chimeric unique bacterial sequence reads were obtained after quality filtering (Table S3.3). Following taxonomic classification, a total of 9 different phyla with a mean relative abundance ≥1 % were identified (Figure 3.3). The most dominant bacterial phyla detected over all the samples included OTUs from the Actinobacteria (66 %), Proteobacteria (23 %) and Acidobacteria (6 %). The Proteobacteria was dominated by the Alphaproteobacteria (13 %), followed by the Gamma- (6 %), Beta- (3 %) and Deltaproteobacteria (1 %). Furthermore, Acidobacteria, Bacteroidetes, Deltaproteobacteria and Gemmatimonadetes were detected in higher relative abundance during the wet season.

Indicator OTU analysis showed that the majority of indicator OTUs belonged to the three most abundant bacterial phyla detected in all soil samples and included the Acidobacteria (Acidobacteriaceae), Actinobacteria (Actinomycetales, AKIW543), and Proteobacteria (Acetobacteraceae, Bradyrhizobiaceae, Burkholderiaceae Caulobacteraceae, Hyphomicrobiaceae, Methylobacteriaceae, Pseudomonadaceae, Xanthobacteraceae, Xanthomonadaceae) (Table 3.2 and 3.3). Only two of the indicator taxa belonged to less abundant phyla, the Bacteriodes (Sphingomonadaceae) and Firmicutes (Bacillaceae). The indicator species analysis showed that OTUs from the Actinomycetales were the most abundant in samples of both soil fractions (bulk and rhizosphere soil) and seasons (wet and dry).

Indicator OTUs from the Acidobacteriaceae, Bradyrhizobiaceae, Caulobacteraceae and Xanthomonadaceae were more frequently detected at a higher relative abundance in rhizosphere soil samples collected in the wet season, while Pseudomonadaceae occurred more frequently in rhizosphere soil samples collected in the dry season. Indicator OTUs from the Sphingomonadaceae occurred in higher frequencies in both soil fractions during the wet season. Furthermore, indicator analysis showed that OTUs from the Xanthobacteraceae were more frequently detected in the rhizosphere soil samples and Methylobacteriaceae in the bulk soil

samples. Acetobacteraceae and Methylobacteriaceae were detected in both seasons, whereas AKIW543 (uncultured bacterial clone), Bacillaceae and Burkholderiaceae were indicators for the wet season samples and Hypomicrobiaceae for the dry season samples (Figure 3.4 and Figure 3.5).

4.3 Correlations between bacterial taxa and soil chemical variables

A total of 12 bacterial taxa showed significant correlations (Table S3.4) with the soil physico-chemical variables (Figure 3.6). Three of these taxa, Bradyrhizobiaceae, Pseudomonadaceae and Xanthomonadaceae, were also identified as indicator taxa. Positive correlations were observed between Pseudomonadaceae and the Na^+, K^+, altitude, H^+, NH_4^+, NO_3^- and pH levels. However, Pseudomonadaceae showed negative correlations with all the other variables as well as all the other bacterial taxa. Bradyrhizobiaceae and Xanthomonadaceae showed positive correlations with the C, C:N, resistance, Mg^{2+} and Ca^{2+} levels and correlated negatively with Na^+, K^+, NO_3^- and K/Na levels. This was predominantly the case for all the other bacterial taxa. In addition, strong negative correlations were seen between the resistance in the soil and all soluble ions measured. Monovalent and divalent ions, respectively, showed strong positive correlations with each other. Furthermore, pH showed a strong negative correlation with the H^+ and positive correlation with Ca^{2+}.

5. Discussion

Soil environments host highly diverse and complex bacterial communities which play an important role in soil nutrient cycling and plant productivity (Richards et al. 1997; Van der Heijden et al. 2008). In this study, we used high throughput sequencing to elucidate the composition and factors that can affect bacterial communities associated with *A. linearis*. We focused on the diversity, taxonomic classification and relative abundance of bacterial communities. Furthermore, we investigated the correlations between bacterial taxa and soil chemical variables (Barberán et al. 2014; Figuerola et al. 2015; Smit et al. 2001).

Results from this study showed that the bacterial communities in the bulk soil were more diverse than rhizosphere communities which support the results of other

studies (Dennis et al. 2010; Uroz et al. 2010). However, this was only true in this study for samples collected in the dry season. No differences were observed in α-diversity and β-diversity between bulk and rhizosphere soil collected in the wet season (Figure 3.1 and Figure 3.2). A possible explanation for this observation could be that there was an increase in soil water availability during the wet season. Therefore, soil particles, including nutrients and microbes, could be transported by water through pores and channels in the soil (Abu-ashour et al. 1994; Horn et al. 1994). Fynbos soil is very sandy and results in relative rapid movement of water through the soil (Muofhe & Dakora, 2000). As a result, nutrients normally restricted to the rhizosphere area, could have been transported to bulk soil areas (Abu-ashour et al. 1994). Therefore, this influx of nutrients to previously oligotrophic areas might have changed the bacterial community composition of the bulk soil to resemble those communities closer to the rhizosphere.

No evidence was found to support our hypothesis that agricultural activities affects microbial communities associated with these fynbos plants. This may indicate that *A. linearis* plant rhizospheres are able to influence bacterial communities that are not affected by these two factors (Doornbos et al. 2011; Haichar et al. 2014). However, as hypothesized, the sampling season had a great effect on the structure of the bacterial communities (Figure 3.2). Many factors can directly or indirectly contribute to this change in bacterial communities between seasons and we considered four possible explanations. Soil water availability may play an important role in not only affecting the diversity, but also the structure of bacterial communities (Stevenson et al. 2014). Another important factor could be soil temperature, which is also directly affected by the change of seasons. During the dry season fynbos soil can reach very high temperatures. Conversely, in the wet season temperatures can drop very low (Cowling et al. 1996; Van Wilgen et al. 2012). It is well known that temperature affects microbial activity and structure (Pietikäinen et al. 2005). Seasonal change may also influence the chemical properties of soil (Stevenson et al. 2014). In the wet season, higher C and C/N rations were detected (Figure 3.2) which were likely due to an increased abundance and degradation of plant material (Denef et al. 2001). The complex physiological functioning of plants is also affected by seasonal change (Aulakh et al. 2001). Consequently, root exudation by *A. linearis* could have differed

between the wet and dry seasons, affecting the structure of the soil bacterial communities, and this would need to be investigated in future studies.

The most abundant bacterial taxa detected in this study were the Acidobacteria, Actinobacteria and Proteobacteria (Figure 3.3) which is consistent with findings of others (Leff et al. 2015; Postma et al. 2016; Smit et al. 2001). Furthermore, the majority of indicator taxa identified between different sampling seasons (Figure 3.4) and soil fractions (Figure 3.5) were also members of these three dominant phyla. Only two of the indicator taxa belonged to the less dominant Bacteriodes and Firmicutes. This supports the hypothesis of Fortunato et al. (2013), which states that dominant bacterial groups play an important role in shaping the community structure, and indicates that variability in an environment is driven by changes in the most abundant taxa.

Three of the indicator taxa showed significant correlations with the soil chemical variables (Figure 3.6). These taxa belong to the highly abundant phylum Proteobacteria and included the families Bradyrhizobiaceae, Pseudomonadaceae and Xanthomonadaceae. Some members of the Pseudomonadaceae are known to promote plant growth, degrade pesticides and suppress disease, although other members are known to be potential plant pathogens (Pesaro & Widmer, 2006). Higher Na^+ and K^+ levels were found to significantly influence the β-diversity of bacterial communities in the dry season. These two cations play important roles in bacterial cells and are taken up through active transport by specialized membrane transporters. Trans membrane transporters are also involved in the adaptation of the cell to osmotic or salt stress (Corratgé-Faillie et al. 2010; Dimroth, 1987). The Pseudomonadaceae, an indicator species of dry rhizosphere soil samples, showed strong positive correlations with Na^+ and K^+. In contrast, Bradyrhizobiaceae and Xanthomonadaceae, identified as indicator species for wet rhizosphere soil samples, correlated negatively with Na^+. Carbon and soil resistance were found to significantly correlate with the β-diversity of the bacterial communities and these two variables correlated positively with Bradyrhizobiaceae and Xanthomonadaceae. Bradyrhizobiaceae are well known to be able to form symbiotic interactions with plant roots to aid in nitrogen fixation (Antoun et al. 1998; Lowther & Heather, 1993). Commonly occurring in soil, some members of the Xanthomonadaceae (*X.*

albilineans and *X. fastidiosa*) are known to cause plant diseases (Pieretti et al. 2009).

6. Conclusion

The CFR is characterized by warm, dry summer and cold, wet winters. These seasonal changes appeared to have the largest effect on bacterial communities associated with *A. linearis* plants. Bacterial communities associated with the agricultural systems were highly similar to that of the natural systems. This was also the case for communities between different farms. These findings support the idea that *A. linearis* plants are able to influence bacterial communities, possibly through a mechanism involving root exudate composition. Additionally, this communication between plant and bacteria was not affected by commercialization or the location of *A. linearis* plants. To our knowledge, this work represents the first effort to get a better understanding on bacterial communities associated with the commercially important fynbos plant, *A. linearis*.

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Figures and Tables

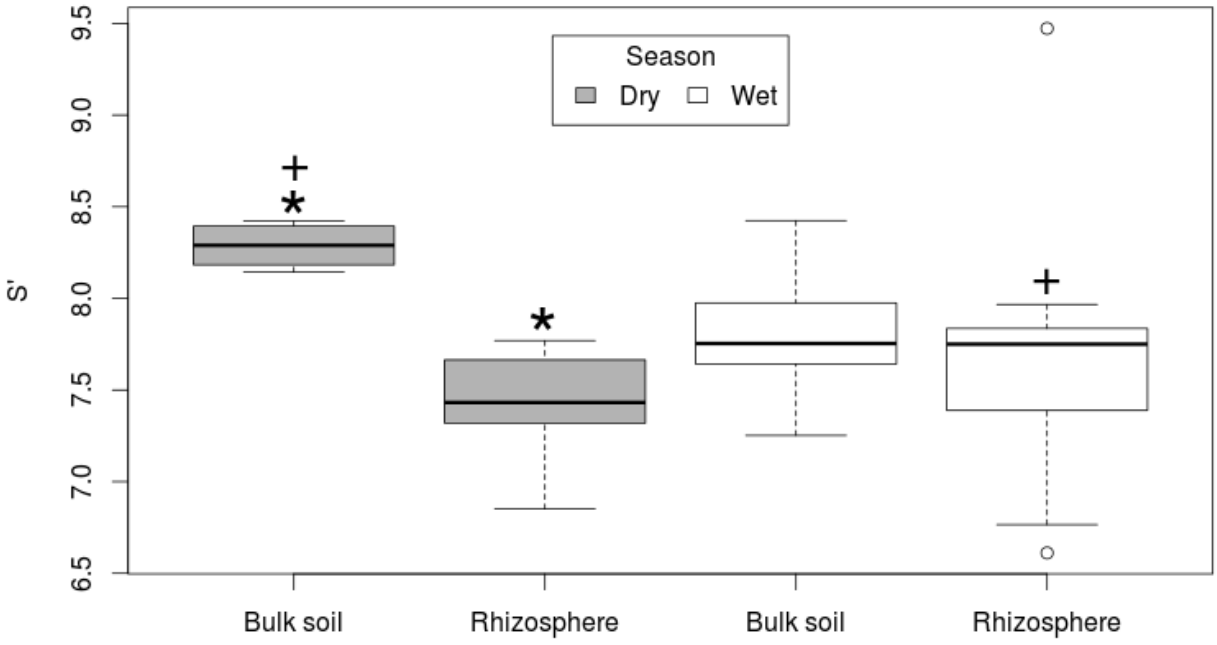

Figure 3.1: Shannon diversity index (S') for bacterial communities associated with soil collected in the dry and wet seasons. Bacterial communities associated with the bulk soil collected in the dry season showed significant difference to rhizosphere soil collected in both dry (*: p = 0.0010650) and wet (+: p = 0.0166159) seasons. Significance between samples was determent using ANOVA and Turkey honestly significant difference post hoc test.

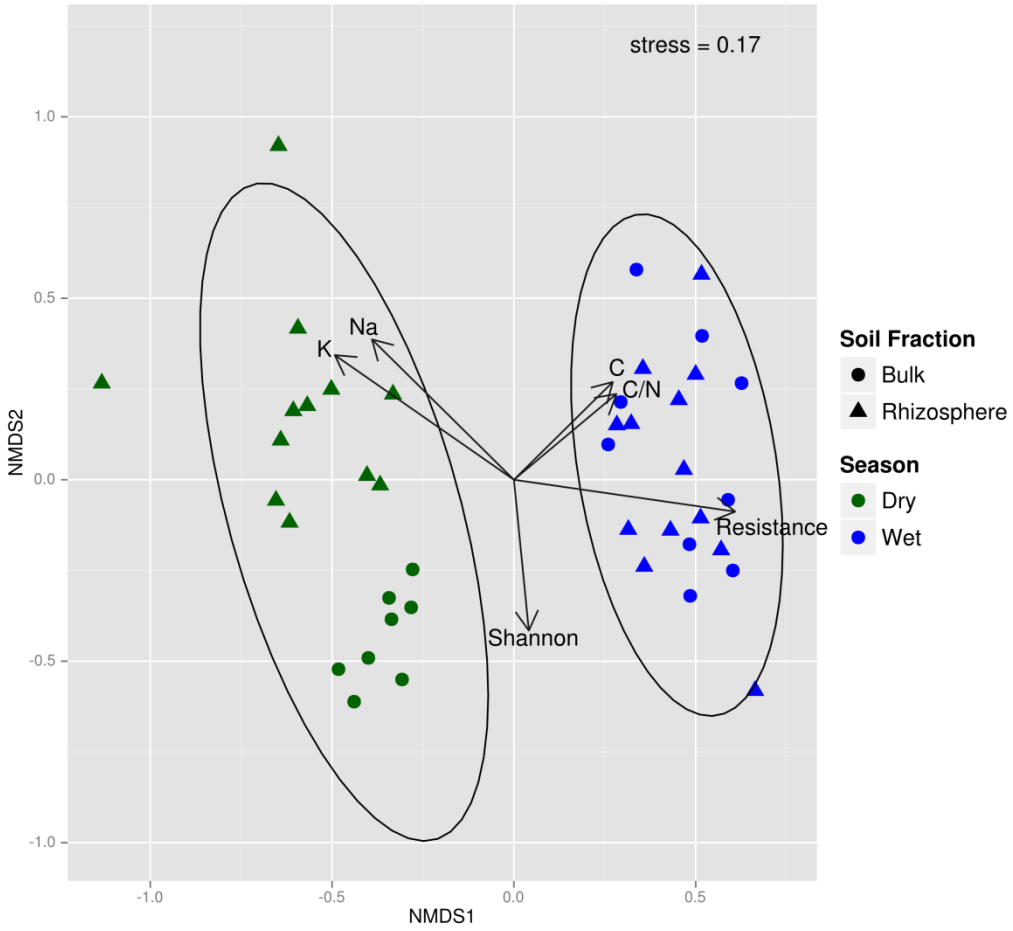

Figure 3.2: Non-metric multidimensional scaling ordination plot of bacterial communities based on the Bray-Curtis distance. Soil chemical variables that have a significant correlation ($p < 0.05$) with the bacterial communities are indicated with the overlaid bi-plot. Ellipses represented the samples which were within 95 % confidence limit and showed a significant difference between the dry and wet season (Stress = 0.17).

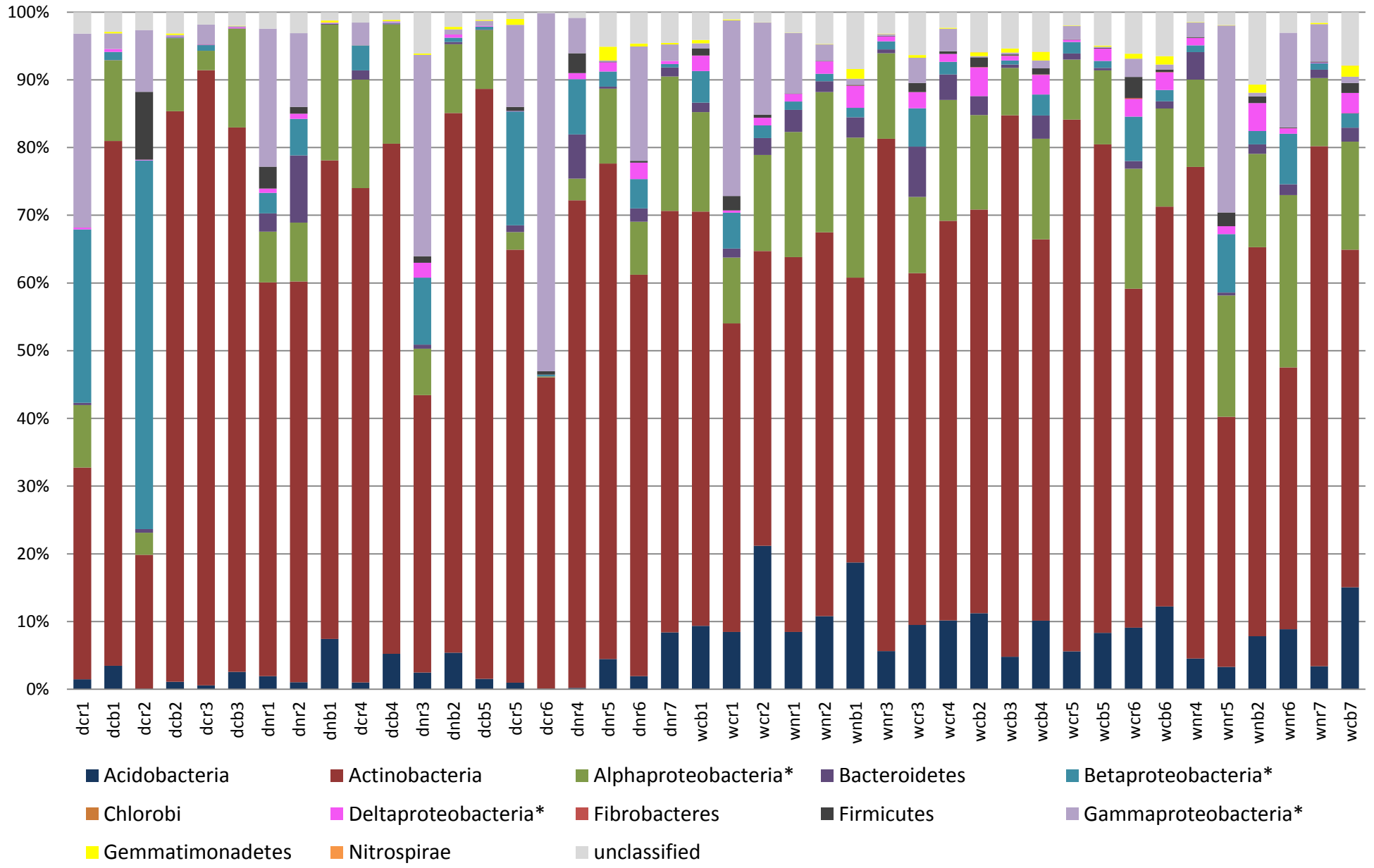

Figure 3.3: Relative abundance of classified sequences at phylum and class (*) level within all the samples. Only sequences with a homology of ≥ 80 % and relative abundance of ≥ 1 % were included.

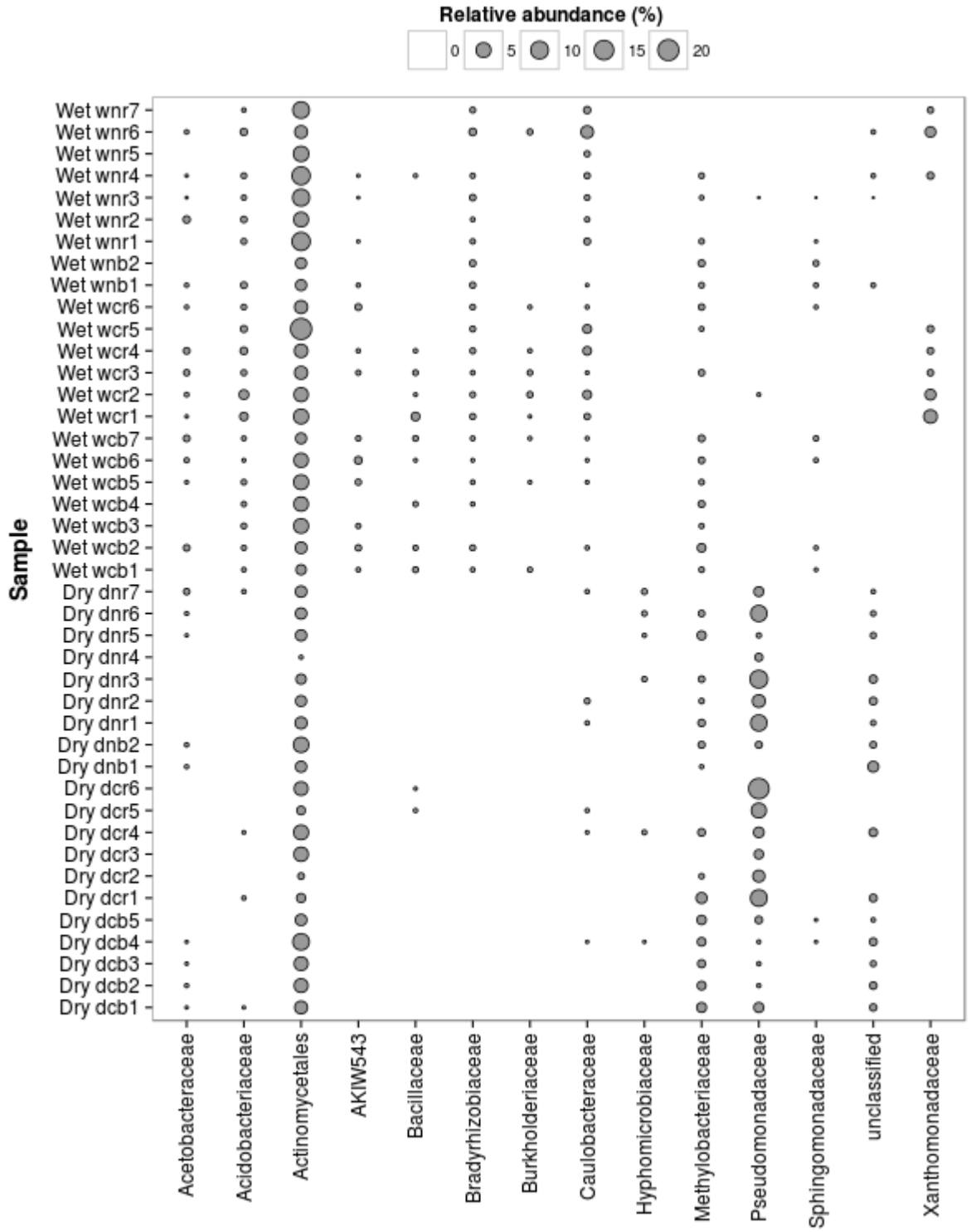

Figure 3.4: Bubble plot of indicator OTUs between wet and dry sampling season for each soil sample. Indicator OTUs were classified up to order/family level. Only OTUs with significant indicator values ($p \leq 0.05$) were shown. Relative abundance (%) of indicator OTUs are indicated by the size of each bubble.

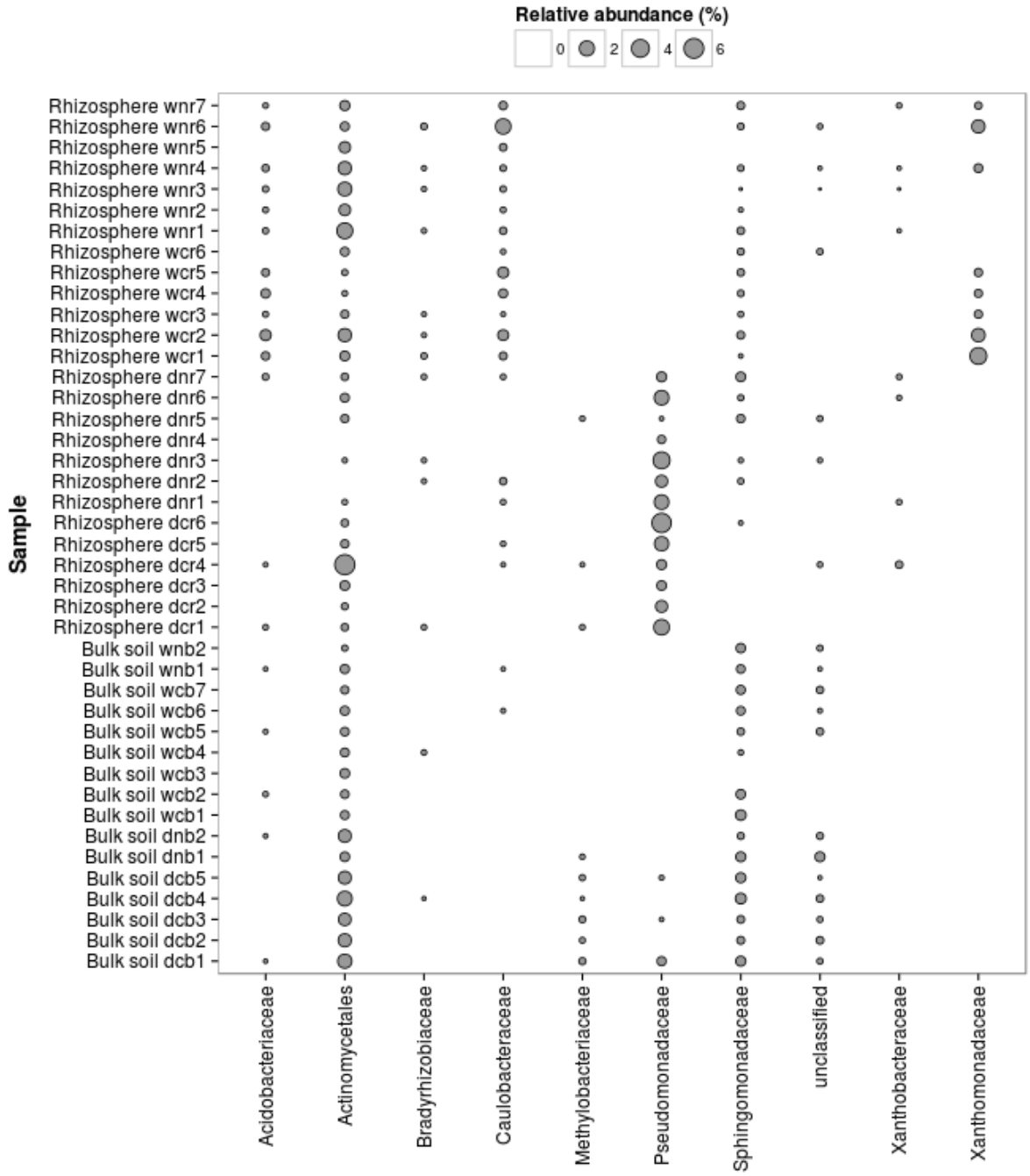

Figure 3.5: Bubble plot of indicator OTUs between rhizosphere and bulk soil for each sample. Indicator OTUs were classified up to order/family level. Only OTUs with significant indicator values ($p \leq 0.05$) were shown. Relative abundance (%) of indicator OTUs are indicated by the size of each bubble.

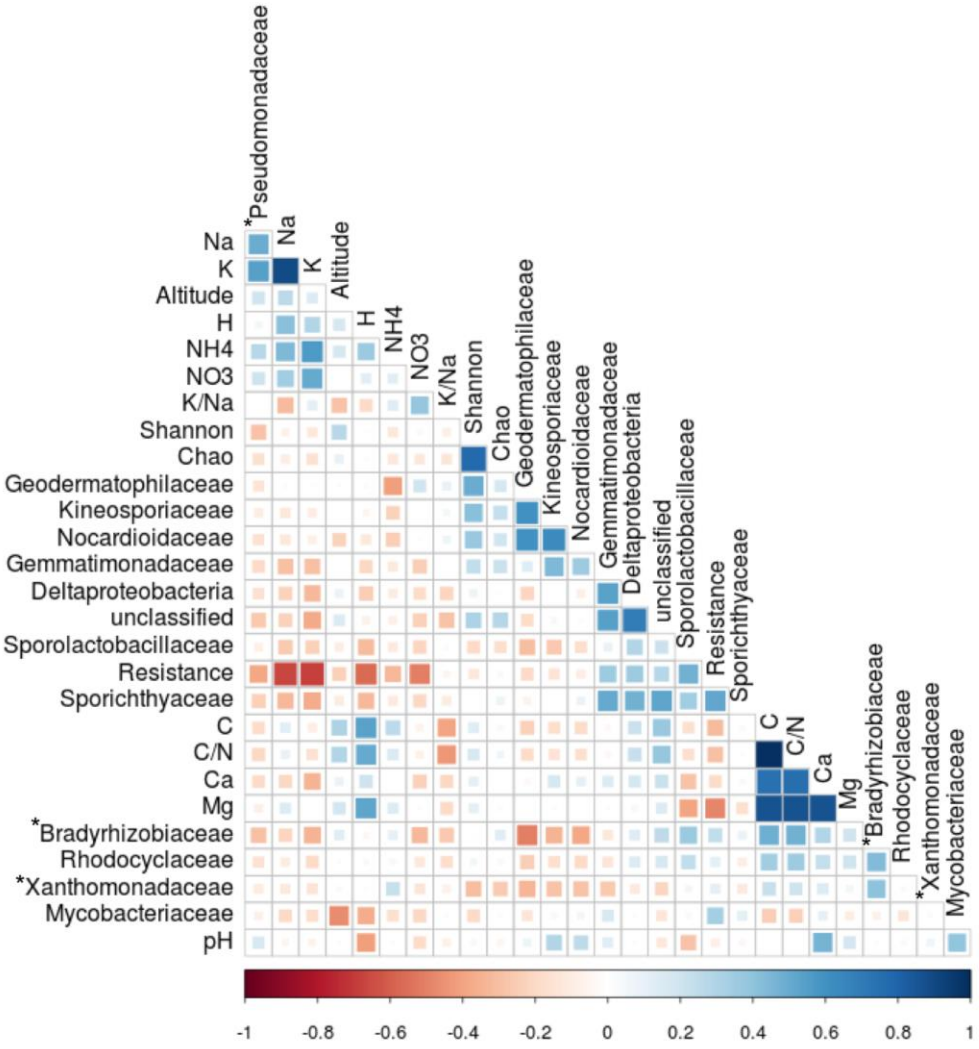

Figure 3.6: Correlogram of spearman correlations ($r \geq 0.5$ and $p < 0.05$) between bacterial taxa and soil chemical variables. Only bacterial taxa that showed correlations with the soil chemical variables were indicated. Taxa were classified up to the highest classification. Marked bacterial groups (*) were also identified as indicator taxa (Figure 3.4 and Figure 3.5.).

Table 3.1: Sampling sites information

Sample name	Soil fraction	GPS coordinates	Altitude (m)	Type	Season	Farm
dcb5	Bulk soil	32.449 S 18.86485 E	679	Commercial	Dry	Kleinvlei
dcr5, dcr6	Rhizosphere					
dnr5	Bulk soil	32.451916 S 18.854 E	595	Natural		
dnr6, dnr4, dnr7	Rhizosphere					
wcb1	Bulk soil	32.449 S 18.86485 E	681	Commercial	Wet	
wcr1, wcr2	Rhizosphere					
wcb2	Bulk soil	32.45593 S 18.858 E	632			
wcr3, wcr4	Rhizosphere					
wnb1	Bulk soil	32.451916 S 18.854 E	595	Natural		
wnr1, wnr2, wnr3	Rhizosphere					
dcr1	Rhizosphere	32.0479 S 18.9916666 E	471	Commercial	Dry	Klipopmekaar
dcb1	Bulk soil					
dcr2	Rhizosphere	32.04708333 S 18.988666 E	457			
dcb2	Bulk soil					
dcr3	Rhizosphere	32.05725 S 18.957166 E	512			
dcb3	Bulk soil					
dcr4	Rhizosphere	32.06005 S 18.995233 E	499			
dcb4	Bulk soil					
dnb1	Bulk soil	32.0663 S 18.980 E	444	Natural		
dnr1, dnr2	Rhizosphere					
dnr3	Rhizosphere	32.0632 S 19.01205 E	580			
dnb2	Bulk soil					
wcb3 *	Bulk soil	32.0413 S 18.99795 E	482	Commercial	Wet	
wcb4 *	Bulk soil	32.000 S 18.0166325 E	426			
wcr5	Rhizosphere	32.047366 S 18.993 E	474			
wcb5	Bulk soil					
wcr6	Rhizosphere	32.047 S 18.98665 E	451			
wcb6	Bulk soil					
wcb7 *	Bulk soil	32.056 S 19.0062 E	538			
wnb2	Bulk soil	32.0662 S 18.9805 E	445	Natural		
wnr4, wnr5, wnr6, wnr7	Rhizosphere					

* Only bulk soil samples were available for these locations

Table 3.2: Indicator OTUs with significant indicator values $p \leq 0.05$ for wet and dry sampling seasons, classified up to order/family level

Taxonomic classification		OTU number								
Phylum	**Order/Family**									
Proteobacteria	Acetobacteraceae	OTU000182	000249	000919						
Acidobacteria	Acidobacteriaceae	OTU000113	000137	000267	000379	000427	000447	000699	000962	000001
Actinobacteria	Actinomycetales	OTU000003	000007	000009	000013	000015	000018	000019	000021	000024
		OTU000025	000026	000027	000029	000030	000032	000039	000043	000046
		OTU000047	000050	000053	000056	000057	000058	000062	000064	000066
		OTU000067	000074	000076	000078	000080	000081	000082	000085	000086
		OTU000092	000095	000096	000097	000107	000117	000118	000122	000123
		OTU000130	000136	000142	000150	000151	000159	000162	000167	000169
		OTU000170	000173	000176	000181	000183	000188	000189	000191	000193
		OTU000195	000197	000208	000210	000213	000217	000218	000219	000229
		OTU000233	000234	000247	000253	000254	000258	000260	000264	000265
		OTU000266	000268	000275	000276	000281	000282	000308	000309	000313
		OTU000318	000325	000327	000340	000342	000343	000354	000356	000357
		OTU000367	000383	000410	000423	000426	000431	000434	000449	000454
		OTU000469	000481	000483	000508	000561	000569	000572	000601	000612
		OTU000616	000620	000684	000688	000721	000730	000745	000748	000763
		OTU000765	000775	000785	000792	000803	000829	000837	000849	000871
		OTU000876	000887	000895	000923	000973	001145	001159	001191	001253
		OTU001346	001628	001672						
Actinobacteria	AKIW543	OTU000336	000396	000993	001265					
Firmicutes	Bacillaceae	OTU000203	000226							
Proteobacteria	Bradyrhizobiaceae	OTU000106	000221	000347	000399	000432	000872	001370	000302	
Proteobacteria	Caulobacteraceae	OTU000028	000225	000364	000391	000428				
Proteobacteria	Hyphomicrobiaceae	OTU000452								
Proteobacteria	Methylobacteriaceae	OTU000048	000090	000124	000128	000139	000172	000246	000305	000414
		OTU000435	000593	000701	000794	000813	000976	001256		
Proteobacteria	Pseudomonadaceae	OTU000004	000011	000023	000031	000040	000042	000051	000102	000121
		OTU000129	000131	000138	000147	000149	000192	000199	000200	000211
		OTU000230	000243	000262	000270	000283	000288	000294	000296	000316
		OTU000322	000346	000370	000395	000433	000608	000756	000893	000915
		OTU001198								
Bacteriodes	Sphingomonadaceae	OTU000398								
Unclassified	Unclassified	OTU000037	000103	000163	000549	000717	000851	001447		
Proteobacteria	Xanthomonadaceae	OTU000016	000054	000201						

Table 3.3: Indicator OTUs with significant indicator values $p \leq 0.05$ for rhizosphere and bulk soil samples, classified up to order/family level

Taxonomic classification		OTU number								
Phylum	**Order/Family**									
Acidobacteria	Acidobacteriaceae	OTU000115	000137	000379						
Actinobacteria	Actinomycetales	OTU000003	000008	000030	000057	000099	000156	000175	000180	000185
		OTU000193	000214	000258	000264	000275	000315	000426	000445	000829
		OTU000923								
Proteobacteria	Bradyrhizobiaceae	OTU000196								
Proteobacteria	Caulobacteraceae	OTU000028	000225							
Proteobacteria	Methylobacteriaceae	OTU000246								
Proteobacteria	Pseudomonadaceae	OTU000011	000023	000102	000129	000138	000147	000149		
Bacteriodes	Sphingomonadaceae	OTU000035	000036	000398	000517	000586				
Unclassified	Unclassified	OTU000163	000273	000549						
Proteobacteria	Xanthobacteraceae	OTU000503	000016	000054						

Chapter 4

Rhizosphere soil fungal communities associated with natural and commercially grown *Cyclopia* spp. and *Aspalathus linearis*

1. Abstract

Fungi contribute greatly to soil biomass and play important roles in ecosystem processes. Despite this, very little is known about the community structures and distribution, especially in the fynbos biome. The aim of this study was to characterize fungal communities associated with two commercially important fynbos plants, *Aspalathus linearis* (rooibos) and *Cyclopia* spp. (honeybush). We furthermore aimed to evaluate the effect agricultural activities have on these fungal communities. Fungal communities were characterized with high throughput sequencing, using the Ion Torrent platform. Total genomic DNA extracted for the rhizosphere soil samples collected in the wet seasons, for both plant species (Chapter 2 and Chapter 3), were used for polymerase chain reaction amplification of the internal transcribed spacer (ITS) region of the fungal rRNA gene region. Results showed that the most abundant fungal phyla that were detected in all soil samples were the Ascomycota and Basidiomycota. Large variability in the composition of fungal communities at order level was detected between the two different plant species. Honeybush samples were mostly dominated by Agaricales, Chaetothyriales and Mortierellales, whereas rooibos samples were dominated by Chaetothyriales, Eurotiales and Helotiales. Furthermore, β-diversity analysis showed that the honeybush samples tended to cluster into commercial and natural groups. This might be due to the differences measured in soil pH between these two groups. This study further contribute to our understanding of the structure of complex fungal communities in fynbos soil and gives a better insight into the effect of agricultural activities on soil fungal communities.

2. Introduction

Fungi play a fundamental role in soil stabilization, functioning of soil ecosystems and plant health (Klein & Paschke, 2004). Some fungal species are known to cause plant diseases that often lead to significant agricultural losses (Damm et al. 2007; Glienke et al. 2013; Strange & Scott, 2005; Tsuge et al. 2013). However, the majority of fungal species provides a valuable service as they are able to compete with plant pathogens, decompose plant and wood debris, aid in plant nutrient uptake and stimulate plant growth (Brien et al. 2005; Gomes et al. 2003). Furthermore, it

was reported that fungi are able to affect, either directly or indirectly, the rhizosphere bacterial composition by altering the root physiology and exudation of the host plant (Barea, 2005; Filion et al. 1999).

Fungi are very effective in colonizing soil environments and although not as diverse as bacteria, they contribute greatly to the soil biomass. This is mainly due to the hyphae of filamentous fungi that are able to bridge gaps between soil particles and grow across surfaces (Boswell et al. 2007). As a result, these fungal hyphae are able to form networks within the soil (Boswell et al. 2007; Porras-Alfaro et al. 2011; Van Der Heijden & Horton, 2009). These networks provide an extremely efficient way to explore for nutrients in soil environments where resources are known to be unevenly distributed (Boswell et al. 2007). One of the most studied groups of fungi able to form these networks is mycorrhizae, which also form mutualistic relationships with plant roots (Van Der Heijden & Horton, 2009). Fungal networks is of great importance in soil ecosystems as it drives communication between plants and other organisms, enhance nutrient uptake in plants and prevent nutrient leaching from the environment (Barto et al. 2012; Van Der Heijden & Horton, 2009).

Despite the importance of fungi in soil ecosystems, very little is known about the community structures and distribution compared to that of bacteria, especially in the fynbos biome. This is mainly due to the difficulties associated in studying these eukaryotic organisms (Aoki et al. 2015; Gweon et al. 2015; Schoch et al. 2012). Traditional culturing techniques used to study soil fungi, only reveal a small part of the complex fungal communities inhabiting this environment (Aoki et al. 2015; Gweon et al. 2015). Moreover, there is a poor correlation between fruiting bodies and other macroscopic structures isolated from the soil through culturing techniques and the true diversity of soil fungal communities (Nilsson et al. 2011).

The recent advancements in high-throughput sequencing have enabled scientists to get a better understanding of the true diversity of soil fungi through sequencing targeted genetic markers directly from soil samples (Gweon et al. 2015). However, less effort has been made to study the markers to describe fungal communities compared to the extensively studied 16S ribosomal RNA gene of bacteria. The internal transcribed spacer (ITS) region was suggested as a suitable marker to study

environmental fungal samples (Bellemain et al. 2010; Schoch et al. 2012). The ITS region of the nuclear ribosomal subunit is located between the small (18S) and large (28S) subunit gene (Gweon et al. 2015). Fungal cells normally contain a large number of ITS copies. This region is, therefore, an ideal target when analysing environmental samples, such as soil, where DNA quantities are relatively low (Bellemain et al. 2010). It should, however, be kept in mind that this region is known to have variability, even within species, but this is normally limited (Nilsson et al. 2008).

The previous two chapters (Chapter 2 and 3) hypothesized that agricultural practices will affect bacterial communities associated with two commercially important fynbos plants, *Aspalathus linearis* and *Cyclopia* spp. However, we showed that bacterial communities associated with commercial and natural plants were very similar, and thus disproved the hypothesis. Clear differences in bacterial communities between samples collected during the different seasons were detected. Additionally, we found evidence that bacterial interactions and activity was higher during the wet season. Whether fungal communities associated with these plants show the same patterns between commercial and natural sites as bacteria, are unknown. Studies have showed that different physico-chemical factors affect soil fungi and bacteria (García-Orenes et al. 2013; Rousk et al. 2010). One such example is the negative effect soil tillage has on fungal communities, which is much greater than for bacteria. This is mainly due to the disruption of filamentous networks formed by fungi in soil environments (García-Orenes et al. 2013). With the above in mind, we hypothesize that the fungal communities associated with *Aspalathus linearis* (rooibos) and *Cyclopia* spp. (honeybush) will be affected by the agricultural activities in this highly endemic fynbos biome. Furthermore, the aim of this study was to characterize and compare fungal communities associated with these two commercially important fynbos plants.

3. Materials and Methods

3.1 Sample collection and physico-chemical analysis

Rhizosphere soil samples of both *Cyclopia* spp. and *Aspalathus linearis* were collected as described in Chapter 2 and 3 respectively. Only the rhizosphere soil samples collected in the wet season (May 2014 and September 2014) were used for the fungal analysis. A total of 26 samples were analyzed, which included 12 *Aspalathus linearis* (6 commercial and 6 natural) and 14 *Cyclopia* spp. (7 commercial and 7 natural) rhizosphere soil samples (Table 4.1). Abiotic soil properties were measured as described by Postma et al. 2016 (Chapter 2) and included soil resistance, pH, and the concentrations of phosphorous, total soil carbon, extractable cations (Na^+, K^+, Ca^{2+} and Mg^{2+}), nitrate and ammonia.

3.2 DNA extractions and Next Generation Sequencing

Total genomic DNA extracted as described in Chapter 2 and Chapter 3 of all rhizosphere soil samples collected in the wet seasons were used for polymerase chain reaction (PCR) amplification. Primers targeting the internal transcribed spacer (ITS) region of the fungal 18S rRNA gene were used for PCR amplification. A set of 26 uniquely barcoded forward primers (ITS1f - CTTGGTCATTTAGAGGAAGTAA) and one reverse primer (ITS4 - TCCTCCGCTTATTGATATGC) was used (Table S4.1) for one-way multiplex sequencing (Ion Torrent Life Technologies, Carls- bad, USA). The total volume of each PCR reaction was 15 µl and contained 7.5 µl 2x HiFi HotStart ReadyMix (Kapa Biosystems, Boston, MA, USA), 0.5 µM each of the ITS1f and ITS4 primer and 50-200 ng DNA template. Amplification conditions consisted of an initial denaturing step at 95 °C for 5 min followed by 35 cycles of 98 °C for 20 s, 65 °C for 15 s and 72 °C for 30 s. The reaction was completed with a final extension at 72 °C 163 for 1 min and the samples were held at 4 °C. Amplified DNA was purified and size selected using the E-Gel®SizeSelect (Life Technologies, Carlsbad, USA) system. DNA concentration and size distribution of the PCR products (expected size about 400 bp) were verified using the Agilent High Sensitivity DNA Kit on the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). After adjusting the concentration of each sample to 10-15 pM, all samples were pooled to be used for emulsion PCR according to the Ion PGMTM 200 XpressTM Template Kit manual (Life Technologies, Carlsbad, USA). After enrichment using the Ion PGM™ Template OT2 400 Kit on the Ion OneTouch™ 2 System, the samples were loaded onto an Ion 316 Chip for unidirectional multiplex

sequencing using the personal genome machine (PGMTM; Ion Torrent, Life Technologies).

3.3 Sequence processing and statistical analysis

Sequences were demultiplexed using the Ion Torrent software TorrentServer version 3.6.2 which simultaneously removed the barcodes and primer sequences. The open source bioinformatics programs MOTHUR v.1.33.3 (Schloss et al. 2009) and PIPITS, an automated pipeline for analysis of fungal ITS sequences (Gweon et al. 2015) were used for all sequence processing and quality filtering. Sequences with an average quality score of 25 and higher, containing homopolymer regions shorter than 10 base pairs and no ambiguous bases were selected for downstream analysis. All non-chimeric, unique sequences greater than 100 bp were aligned to the UNITE reference database (Abarenkov et al. 2010). Sequences were subsequently clustered into OTUs at a 97 % similarity and taxonomically classified at an 80 % similarity cut-off value.

Qiime version 1.8.0 was used to calculate the Shannon and Simpson diversity indices as well as the Chao1 richness index from a normalized OTU relative abundance table. All other calculations and statistical analyses were performed in the R v3.2 software environment (R Development Core Team 2015). Normality of the physico-chemical variables was tested using the Shapiro-Wilk normality test. Differences in variable values between groups were tested using the non-parametric Kruska-Wallis H test. The post-hoc Kruskal Wallis test after Nemenyi was used to test for significant differences between groups of three and more (PMCMR package).

Differences in fungal community composition (β-diversity), between commercial and natural as well as honeybush and rooibos rhizosphere soil, were determined using the generalized UniFrac (GuniFrac) algorithm (Chen, 2012). This algorithm created weighted (α 0.5), unweighted and variance adjusted weighted distance matrices. Subsequently, significance was tested with GPERMANOVA on the three calculated distance matrices and principal coordinate analysis (PCoA) was used to visualize the result. Figure 4.2 was created with ggplot2 (Wickham, 2009). P-values ≤ 0.05 were considered as significant difference between groups.

4. Results

4.1 Physico-chemical analysis

Certain soil physico-chemical variables differed significantly between honeybush and rooibos samples (Table 4.2). For instance, significantly higher Mg^{2+} concentrations were detected in all honeybush soil samples compared to commercial rooibos soils (Figure 4.1a). Also, Ca^{2+} concentrations were significantly higher in natural honeybush compared to commercial rooibos soil (Figure 4.1b). Consequently, the resistance in rooibos soil samples were significantly higher (Table 4.2). Furthermore, C percentages were higher in commercial honeybush soil compared to all rooibos samples (Figure 4.1d). Interestingly, the only difference between commercial and natural samples was that of the honeybush rhizosphere soil (Figure 4.1c). Significant higher pH values were detected in commercial honeybush soil samples (pH average 4.7 ± 0.53) compared to natural samples (pH average 3.9 ± 0.37).

4.2 Fungal diversity and species richness

No significant differences were detected in the Shannon diversity and Choa 1 total species richness indices between natural and commercial samples collected from honeybush and rooibos plants. However, significant differences (GPERMANOVA F-model = 1.517; p = 0.044) in β-diversity between honeybush and rooibos samples were detected (Figure 4.2). Moreover, the honeybush data points appeared to cluster into commercial and natural groups, with the exception of two commercial samples (hc4b and hc4c) that clustered together with the natural samples. Physico-chemical variables that were found to significantly influence fungal communities include K^{+}, Mg^{2+} and NO_3^{-} levels that were higher in commercial honeybush samples (Table S4.2).

4.3 Fungal community composition

OTUs were classified to 39 fungal orders that belonged to 5 different phyla. The most dominant phyla detected in the honeybush samples included the Ascomycota

(47 %), Basidiomycota (41 %) and Zygomycota (11 %). The Chytridiomycota, Glomeromycota and unidentified fungi contributed less than 1 % of the total number of classified sequences. Rooibos samples were dominated by the Ascomycota (75 %), followed by the Basidiomycota (16 %) and Zygomycota (4 %). The remaining sequences were classified as Chytridiomycota, Glomeromycota and unidentified fungi (5 %) (Figure 4.3a).

At the order level, there was a large variability in the composition of fungal communities between the samples (Figure 4.4). Honeybush samples were mostly dominated by Agaricales (22 %), Auriculariales (6 %), Chaetothyriales (10 %), Hypocreales (6 %), Mortierellales (12 %) and Pleosporales (8 %) (Figure 4.3b). The fungal taxonomic composition in the rooibos samples were found to be distinct from that of the honeybush samples, with the most abundant orders being Chaetothyriales (15 %), Eurotiales (14 %), Helotiales (21 %) and Hypocreales (5 %) (Figure 4.3c). Fungal orders that were detected in relative equal abundances in both plant species included Chaetothyriales (10 – 15 %), Hypocreales (4 – 5 %) and Tremellales (4 %). Three orders, with a relative abundance $\geq$ 1 %, that were only associated with honeybush samples included the Chaetosphaeriales, Geoglossales and Russulales. The Coniochaetales was the only order with a relative abundance $\geq$ 1 % detected in the rooibos and not in the honeybush samples (Table S4.3).

5. Discussion

With the aid of NGS, scientist are now starting to understand the true complexity and immense diversity of fungi in soil environments (Banerjee et al. 2016; Kemler et al. 2013; Rhodes et al. 2011; Porras-Alfaro et al. 2011). In order to contribute to our understanding of the interactions between soil microbes and fynbos plants, we aimed to characterize fungal communities associated with two commercially important fynbos plant species. Furthermore, this study aimed to evaluate the effect of agricultural activities on soil fungal communities associated with *Aspalathus linearis* and *Cyclopia* spp. We hypothesised that the fungal community structures associated with these plants will be affected by agricultural activities.

Major differences were detected in the β-diversity of fungal communities associated with honeybush and rooibos plant species (Figure 4.2). These two plant species are indigenous to geographically distinct areas of the CFR where notable differences exist in soil composition. For example, soil in the distribution areas of rooibos plants are sandier than soil from the mountain slopes of the Langkloof district where honeybush plants grow (Du Toit et al. 1998; Joubert et al. 2008; Mitchell et al. 1984). It is well known that the physical structure of soil particles can directly affect microbial communities (Lauber et al. 2008; Marschner et al. 2001; Wardle et al. 2004). Additionally, communities can also indirectly be affected though differences in water retention times, soil aeration and movement of nutrients such as K^+, Mg^{2+} and Ca^{2+} through soil particles (Canfield et al. 2010).

In this study we found that rhizosphere soil of honeybush plants contained higher concentrations of the monovalent cations, K^+ and Na^+, as well as divalent cations, Ca^{2+} and Mg^{2+}. Additionally, very high C levels and slightly higher NO_3^- concentrations were detected in honeybush soils (Table 4.2). The higher concentrations of K^+, Mg^{2+} and NO_3^- in soil collected from honeybush, showed to significantly affect fungal community structures (Figure 4.2). Similar results were obtained by Gumiere and co-workers (2016), where variations in fungal communities were best explained by K^+ and Mg^{2+} concentrations in soil. Both K^+ and Mg^{2+} are commonly occurring soil elements. Potassium is the most abundant cation in the cytoplasm of all living cells (Corratgé-Faillie et al. 2010), whereas Mg^{2+} is an essential macronutrient required for the growth in plants (Xiao et al. 2014). Magnesium can affect the occurrence of specific soil fungi differently. An example is occurrence of arbuscular mycorrhizal fungal genera *Glomus* and *Acaulospora*, which are often associated in soil with high Mg^{2+} concentrations. In contrast, genera such as *Scutellospora* and *Gigaspora* are associated in soil with low Mg^{2+} concentrations (Gryndler et al. 1991). Furthermore, increased Mg^{2+} concentrations are often associated with an increase in Ca^{2+} concentrations which was also the case in this study (Gryndler et al. 1991).

Differences detected in fungal communities associated with the rhizosphere soil of honeybush and rooibos, could also be due to the different root cell components and root exudates associated with the respective plant species (Haichar et al. 2014;

Marschner et al. 2001). Plants release species specific root exudates into the rhizosphere soil. These molecules serves as communication signals to initiate symbiotic interactions with specific microorganisms. A well studied research field is the interactions between fynbos plants and mycorrhizal fungi (Cloete et al. 2007; Dakora & Phillips, 2002; Lotter et al. 2014; Power et al. 2010; Spriggs & Dakora, 2009; Spriggs et al. 2003). Many Fabaceae plant species, that includes honeybush and rooibos species, form mycorrhizal symbiotic interactions (Power et al. 2010; Lotter et al. 2014; Spriggs & Dakora, 2009). Plants in the nutrient limiting fynbos soil environments depend on these interactions to increase acquisition of nutrients, especially N and P (Cloete et al. 2007; Dakora & Phillips, 2002). The most dominant order associated with the rooibos samples were the Helotiales. This dominant ectomycorrhiza group of fungi consist of approximately 2000 described species. However, it contains the largest number of undescribed root-associated fungi (Tedersoo et al. 2009).

Apart from the differences in soil and plant physiological properties, agricultural disturbances may also affect the fungal communities in fynbos soil. Supporting our hypothesis, the β-diversity analysis showed that the honeybush samples tended to cluster into commercial and natural groups (Figure 4.2). Soil pH was the only physico-chemical variable that showed to be significantly different between these groups (Figure 4.1). Therefore, differences in soil pH between commercial and natural honeybush plants might be one of the main reasons why changes in fungal communities were detected. However, weak correlations between fungal community structures and soil pH often exist due to the generally wide pH ranges for optimal growth (ranging from pH 5 – 9) (Rousk et al. 2010). On the other hand, fynbos soils are known to be acidic (pH values below 5) (Richards et al. 1997). Consequently, we hypothesize that the correlations between soil pH and fungal communities are stronger when pH values are below 5.

In agreement with many other studies, the most dominant fungal phyla detected in all soil samples were the Ascomycota and Basidiomycota (Figure 4.3a) (Aoki et al. 2015; Brien et al. 2005; De Castro et al. 2016; Hartmann et al. 2015). Fungal orders that were detected in both honeybush and rooibos soil samples included the Chaetothyriales, Hypocreales and Tremellales. Members from Chaetothyriales are

commonly occurring facultative root endophytic order (Tedersoo et al. 2009). Hypocreales include genera such as *Fusarium*, *Ilyonectria* and *Clonostachys* which are frequently isolated from soil environments (Porras-Alfaro et al. 2011). The order Tremellales contains unicellular fungi that include the well known genera *Cryptococcus* and *Dioszegia*. Generally, this order favour wetter soils with a lower pH (Vishniac, 2006) which is in agreement with the soil properties found in this study. Honeybush samples were dominated by the species rich mushroom order Agaricales. Additionally, Mortierellales, a wide spread soil fungal order also occurred in relative high abundance in these samples. Some species in this order are known to solubilise phosphate (Zhang et al. 2011) and might play an important role in phosphate cycling in the nutrient limited fynbos soils. Interestingly, the order Geoglossales were only detected in honeybush rhizosphere soil. It is hypothesized that this tongue shaped fungal order could be associated with various plant species (Loizides et al. 2015). However, the ecology of this group is not fully understood and potential plant hosts have not yet been established (Hustad et al. 2013). We, therefore, hypothesize that honeybush plants may serve as a potential host for Geoglossales species, and this could form the basis future studies.

6. Conclusion

According to our knowledge, this is the first study done to characterize fungal communities associated with commercially important fynbos plant species, *Aspalathus linearis* and *Cyclopia* spp. Significant differences were detected between fungal communities associated with rhizosphere soils collected from these two plants species. Our hypothesis was rejected when considering the structure of fungal communities associated with rooibos rhizosphere soil, since fungal communities were highly similar between natural and commercially grown plants. However, we found some evidence that support our hypothesis with fungal communities associated with honeybush rhizosphere soil. Major differences in soil pH between natural and commercially grown honeybush plants appeared to influence fungal communities. Future studies should, therefore evaluate the correlations between soil pH and fungal communities in acidic soil environments. Finally, this study found evidence suggesting that honeybush plants may serve as a potential host for Geoglossales species.

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Figures and Tables

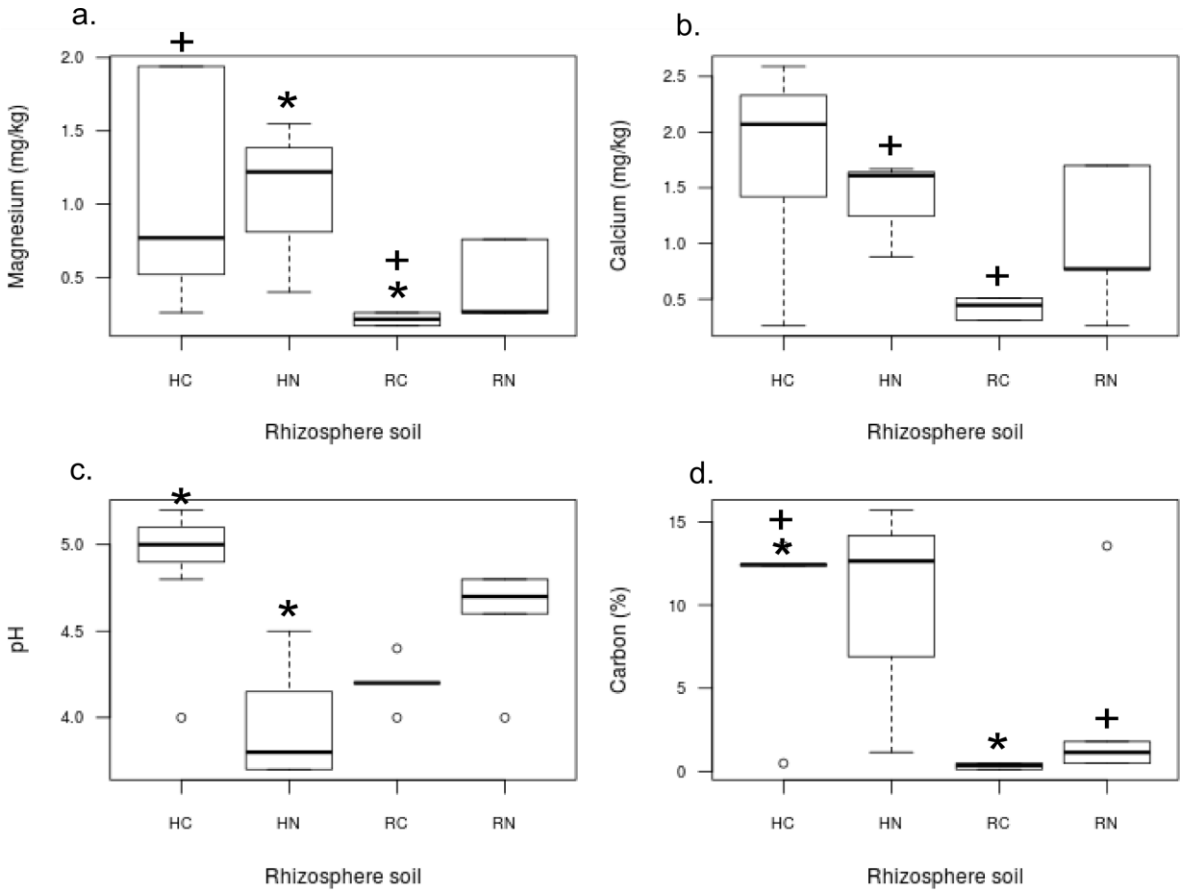

Figure 4.1: Boxplots of physico-chemical variables between soils collected from natural and commercially grown honeybush and rooibos plants (HC: Honeybush Commercial; HN: Honeybush Natural; RC: Rooibos Commercial; RN: Rooibos Natural). a. Mean magnesium concentrations (* p = 0.003; + p = 0.004). b. Mean calcium concentrations (+ p = 0.009). c. Mean pH values (* p = 0.001). d. Mean carbon percentages (* p = 0.018; + p = 0.002).

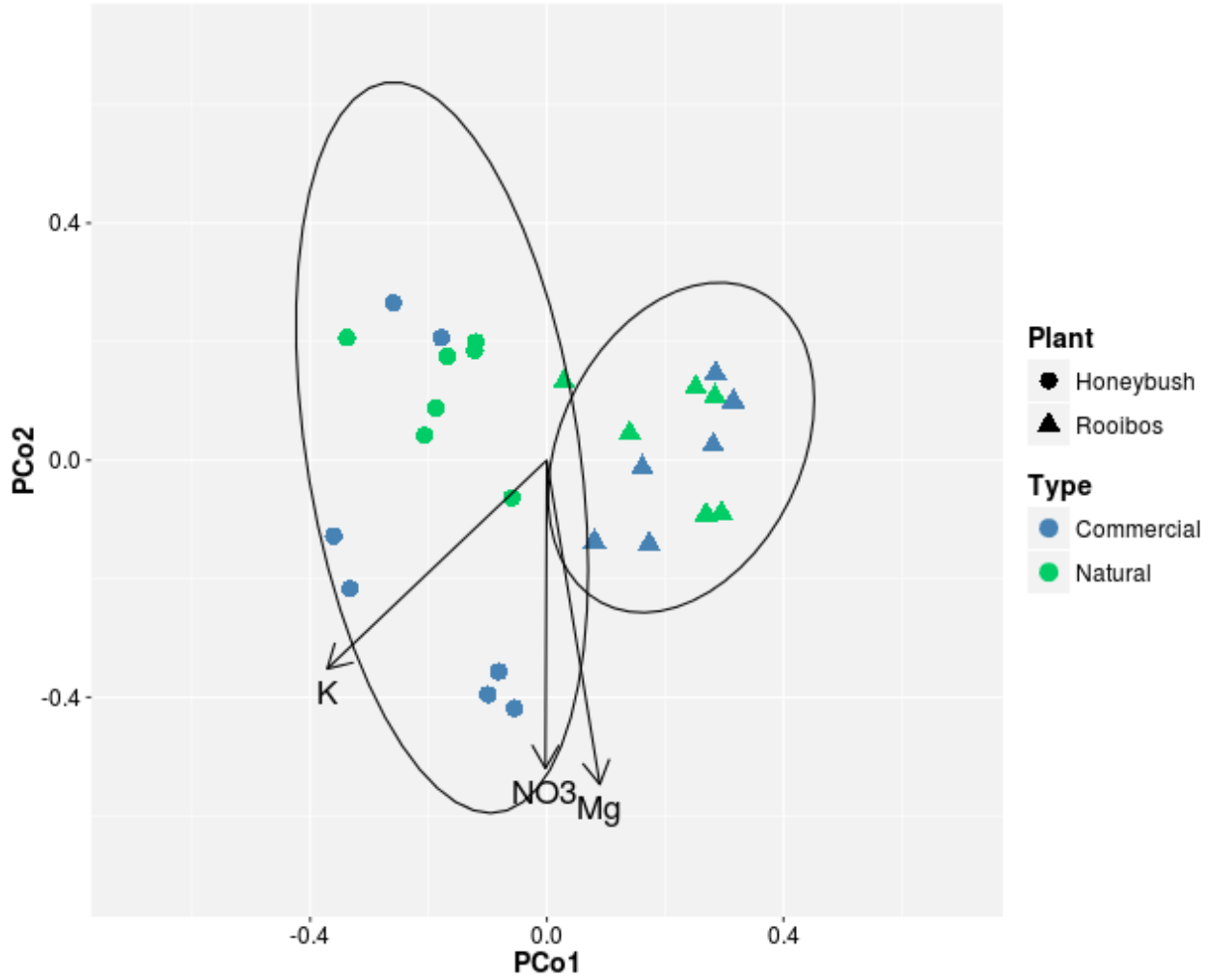

Figure 4.2: Principle coordinate analysis bi-plot of the generalized UniFrac alfa 0.5 distance matrix of fungal community composition between honeybush and rooibos (GPERMANOVA F-model = 1.517; p = 0.044) as well as natural and commercial samples. The overlaid bi-plot display soil chemical variables that have a significant correlation ($P < 0.05$) with the fungal community structure.

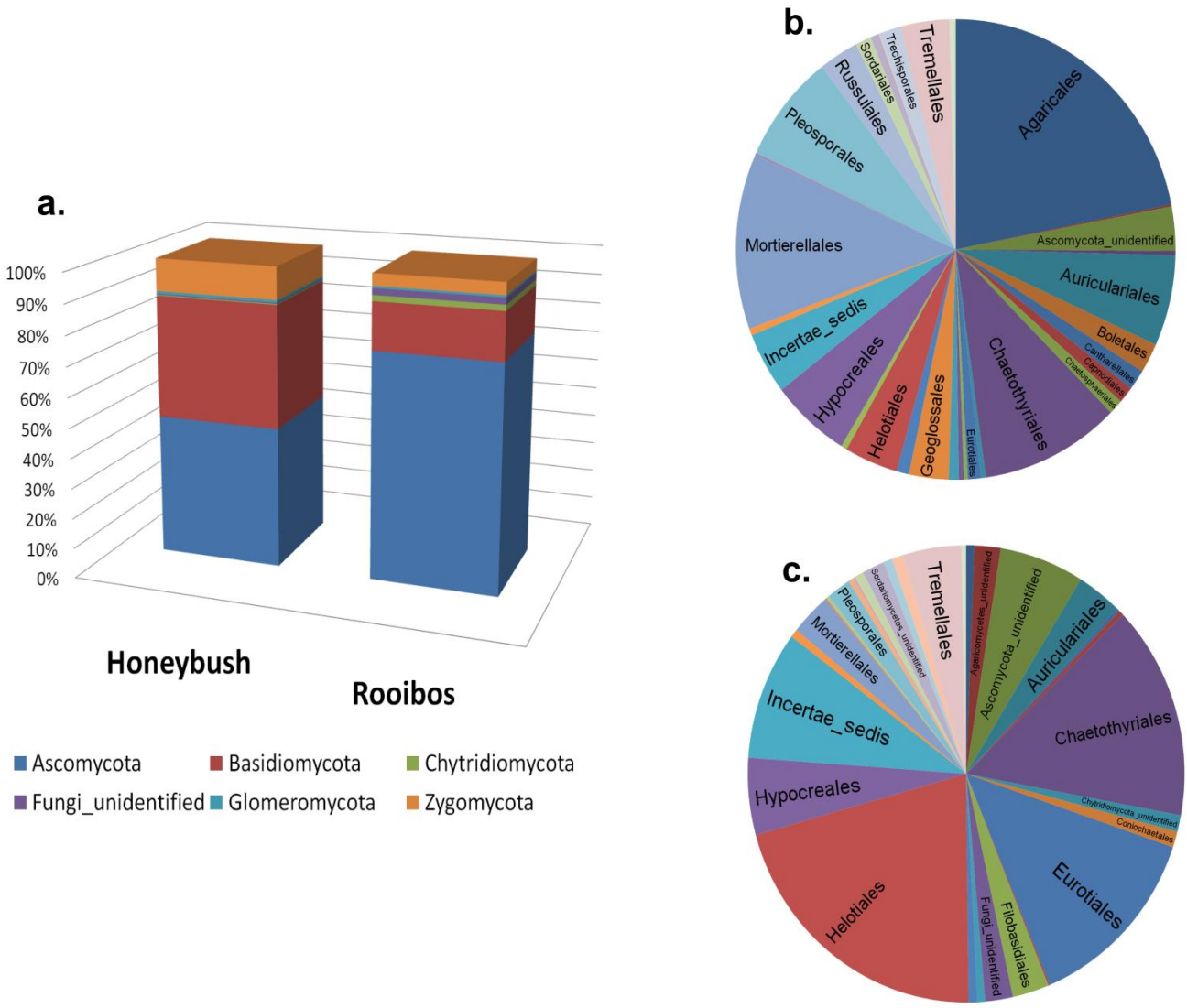

Figure 4.3: a. Relative abundance of classified sequences at phylum level for all honeybush and rooibos samples. b. Relative abundance of classified sequences at order level for honeybush samples. c. Relative abundance of classified sequences at order level for rooibos samples.

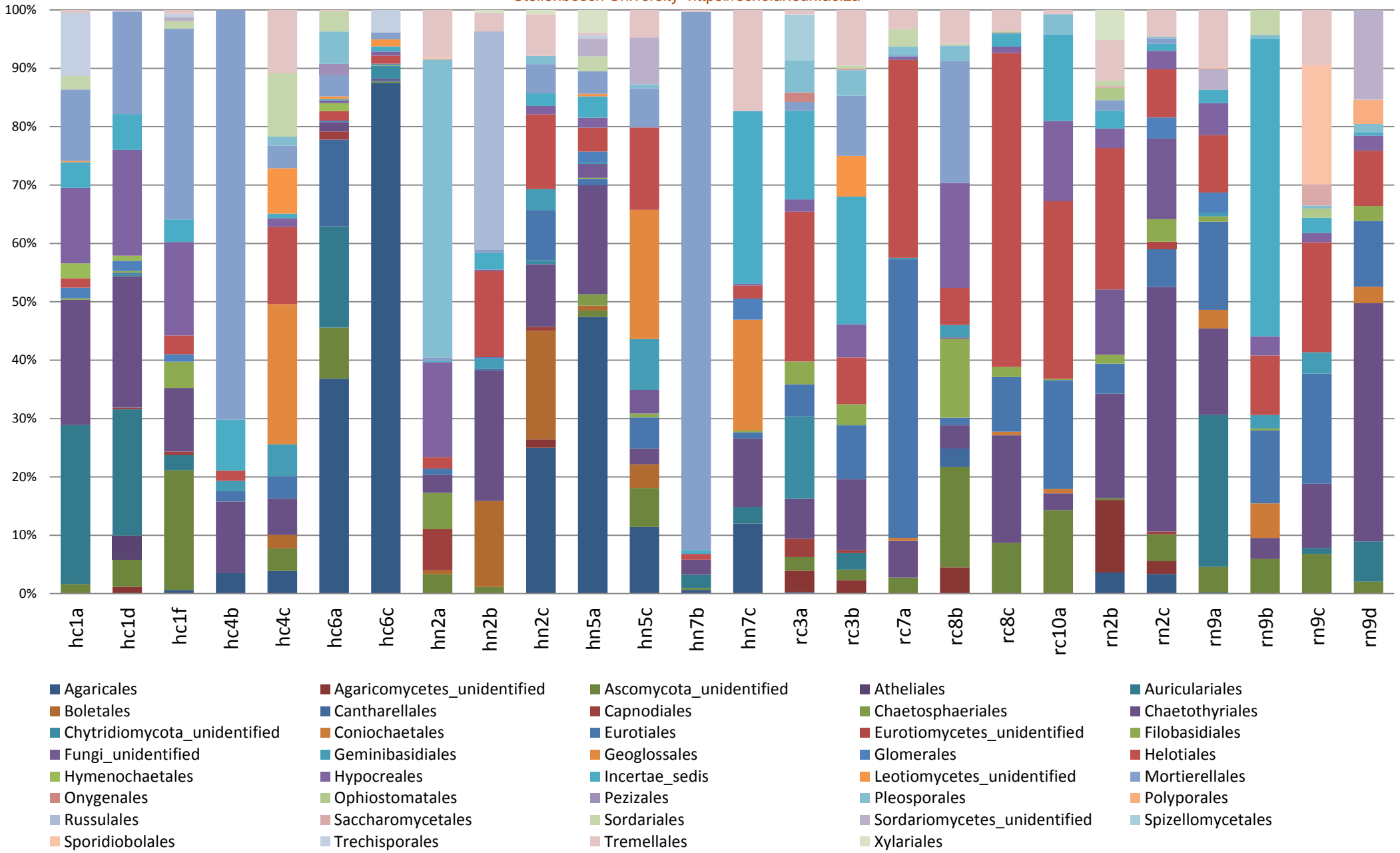

Figure 4.4: Relative abundance of classified sequences at orders level. Only sequences with a homology of ≥80% were used.

Table 4.1: Summary of sampling sites locations, type of soil and plant species for rhizosphere soil samples collected in the wet season

Sample name	GPS	Type	Plant
rc3a, rc3b	S32°27.356minE18°51.496min	Commercial	*Aspalatus linearis* (Rooibos)
rc7a	S32°02.842minE18°59.623min		
rc8b, rc8c	S32°02.866minE18°59.199min		
rc10a	S32°03.361minE19°00.374min		
rn2b, rn2c	S32°27.115minE18°51.251min	Natural	
rn9a, rn9b, rn9c, rn9d	S32°03.972minE18°58.835min		
hc1a, hc1d, hc1f	S34°01.797minE24°.415min	Commercial	*Cyclopia* spp. (Honeybush)
hc4b, hc4c	S33°58.975minE24°12.989min		
hc6a, hc6c	S33°52.131minE23°59.102min		
hn2a, hn2b, hn2c	S34°02.802minE24°20.812min	Natural	
hn5a, hn5c	S33°59.156minE24°12.390min		
hn7b, hn7c	S33°51.545minE23°58.316min		

Table 4.2: Mean concentrations of physico-chemical variables of honeybush and rooibos rhizosphere soil

	Honeybush		Rooibos		Kruskal Wallis Test	
	Mean	SD	Mean	SD	Chi	p-value
Altitude	384.929	167.184	520.083	77.165	2.998	0.083
pH	4.421	0.613	4.400	0.307	0.006	0.938
Re	4638.571	2935.352	10988.333	3944.897	10.929	0.001
H	2.079	2.045	0.664	0.814	3.419	0.064
P	5.286	2.946	4.583	4.602	1.557	0.212
K	71.357	35.487	24.750	9.117	11.978	0.001 *
Na	0.156	0.094	0.060	0.042	9.004	0.003
Ca	1.596	0.688	0.708	0.497	8.072	0.004
Mg	1.104	0.630	0.322	0.208	13.948	0.000 *
C	10.554	5.340	1.716	3.772	11.024	0.001
NO3	1.696	1.944	0.858	0.857	2.244	0.134 *
NH4	7.738	1.282	7.536	0.745	0.024	0.877
shannon	3.278	0.879	3.504	0.706	1.167	0.280
simpson	0.798	0.198	0.838	0.118	0.447	0.504
chao1	33.482	10.890	31.201	8.642	0.320	0.571

□ Significant p-values ($p \leq 0.05$)

* Significant influenced fungal communities

Chapter 5

Concluding remarks and future research

Despite the exceptionally high floral diversity in the Cape Floristic Region (CFR), it has a poor conservation record and many endemic plant species are now classified as endangered (Cowling et al. 2003; Richards et al. 1997; Van Wilgen et al. 2012). One of the main threats to the region's ecosystem is the overexploitation of economically valuable plant species which include rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.). Worldwide, rooibos and honeybush are gaining popularity as herbal teas and subsequently play important roles in supporting rural livelihoods in the Western Cape, South Africa (McKay & Blumberg, 2007). However, overharvesting of natural plants has led to the decline in the populations of these plant species (Du Toit et al. 1998). This decline, together with increasing demand, prompted the establishment of a rooibos and honeybush agriculture industry (McKay & Blumberg, 2007). Establishing these plant species as crops may appear to be a sustainable solution, but it results in the removal of native fynbos and clearing of large tracts of land (Chapin et al. 2000). In addition, as is the case with all monoculture crops, there are incidences of plant pathogens and other problems in the farming of these species. Moreover, Joubert and co-workers (2008) reported on the gradual loss in vigour of commercially grown rooibos and honeybush plants. One of the hypotheses was that this loss in vigour may be linked with changes in bulk soil and root-associated microbial diversity.

Microorganisms play an important role in soil processes (Cotner & Biddanda 2002; Falkowski et al. 2008; Kirk et al. 2004; Torsvik & Øvreås, 2002) and appear to be driving a number of key nutrient cycles in this habitat as well (Chapter 1). Despite this, only a few studies have been done on the microbial diversity in the CFR and little is known on the interactions between microbial communities and fynbos plants (Beukes et al. 2013; Lemaire et al. 2015; Slabbert et al. 2010a; Slabbert et al. 2010b; Stafford et al. 2005; Visagie et al. 2009; Visagie & Jacobs, 2012). How these soil microbial communities change during a disturbance, for example agricultural activities, in this highly endemic region is unknown. Using *A. linearis* and *Cyclopia* spp. as model systems, this study provided the opportunity to characterize and compare the microbial communities of natural and disturbed fynbos systems.

Many challenges arise when studying microorganisms in complex ecological systems such as soil environments (Kirk et al. 2004). One of the most profound

challenges is methodological limitations. Traditional microbial culturing techniques fall short when the true diversity and structure of complex microbial communities are investigated (Kirk et al. 2004). Soil microorganisms live in complex microbial assemblages, interacting with other micro- and macro-organisms, plants and their environment (Van Der Heijden et al. 2008). It is speculated that less than 1% of all microbes can be cultured. Limitations of cultivation-based techniques have led to the use of molecular approaches such as cloning-and-sequencing (Kumar et al. 2006; Kumar et al. 2005), Denaturing Gradient Gel Electrophoresis (DGGE) (Maarit et al. 2001; Niemi et al. 2001; Salles et al. 2004; Stafford et al. 2005; Van Elsas et al. 2000), Terminal Restriction Length Polymorphism (tRFLP) (Berthrong et al. 2013; Liu et al. 1997; Marsh, 1999), Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Slabbert et al. 2010a; Slabbert et al. 2010b) and the most recent technology, Next-Generation Sequencing (NGS) (Kavamura et al. 2013; Kemler et al. 2013). Over the past decade significant progress has been made in NGS technologies and is becoming the method of choice to study microbial community structure and diversity. Different platforms were developed for this massive parallel sequencing, including 454, Illumina, SOLiD and Ion Torrent (Mardis, 2008; Metzker, 2010; Van Dijk et al. 2014). As a versatile and cost effective NGS platform, Ion Torrent has been used in many studies investigating bacterial (Bell et al. 2013; Kavamura et al. 2013; Yergeau et al. 2014; Yergeau et al. 2012) and fungal diversity in soil (Brown et al. 2013; Kemler et al. 2013).

Using the Ion Torrent NGS platform, we aimed to characterize soil microbial communities associated with *A. linearis* and *Cyclopia* spp. Furthermore, we compared microbial communities associated with natural and commercially grown plants and how these communities are affected by the plants root system. Lastly, we investigated the effect different abiotic and physico-chemical factors may have on microbial communities in this unique region.

It is well known that agricultural activities greatly alter soil microbial communities (García-Orenes et al. 2013; Hartmann et al. 2015; Lauber et al. 2013). However, the soil types that have been investigated until now were mostly nutrient rich loam and clay soil types, not the sandy nutrient poor soil found in the fynbos region. This is the first study to investigate the effect of agricultural activities on fynbos soil and the

rhizosphere of the indigenous plants. We hypothesised that the rhizosphere microbial communities associated with naturally occurring *A. linearis* and *Cyclopia* spp. will, like other crops, be affected by agricultural activities. Ultimately we found little evidence to support this hypothesis since the bacterial communities were very similar between natural and commercial plants of both the species we investigated. Furthermore, taxonomic classification (order/family level) of bacterial communities associated with *A. linearis* and *Cyclopia* spp. were dominated by Actinobacteria, followed by Proteobacteria and Acidobacteria. These bacterial community structures appear to be highly similar between both plant species (Chapter 2 and Chapter 3). Unlike the bacteria, significant differences were detected in the taxonomic classification of fungal communities between *A. linearis* and *Cyclopia* spp. plants. This might be due to the differences in physico-chemical properties between the two different types of rhizosphere soils as well as the different plant root physiology that appeared to greatly affect the fungal communities. Similar to the bacterial community analysis of the fungal communities associated with natural and commercially grown *A. linearis* plants also did not reveal any significant difference. However, some evidence supporting our hypothesis was found when considering fungal communities associated with natural and commercially grown *Cyclopia* species. Fungal communities associated with natural and commercially grown *Cyclopia* spp. plants appeared to differ which may be linked to the significant difference in the pH values. Although weak correlations between fungi and soil pH levels ranging from pH 5 – 9 exists (Rousk et al. 2010), we hypothesize that these correlations are stronger in more acidic soils such as in the fynbos (Chapter 4).

Many studies demonstrated the selective and growth promoting effects that rhizodeposition of plants have on soil microbial communities (Grayston et al. 1998; Hartmann et al. 2008; Jones et al. 2004). This plant-driven selection of rhizosphere microbiome for these two fynbos plants appeared to be very strong and was not influenced by agricultural activities. The nutrient poor (Cowling et al. 2003) conditions in the fynbos soil may contribute to the strong effect rhizodeposition of the plants have on structuring microbial communities. Based on these findings we now hypothesise that the rhizosphere effect is more prominent in nutrient poor (oligotrophic) soils compared nutrient rich (copiotrophic) environments.

Given the apparent strong influence fynbos plants have on the soil microbial communities, it stands to reason that although the plants depend on microbial symbiotic associations, the rhizosphere microorganisms are also dependent on the vigour of plants. Plant physiology is highly dependent on seasonality (Aulakh et al. 2001) and physiological changes in the plant, specific in root exudation, are likely to affect the rhizospheric microbiome. Therefore, seasonal change may be indirectly responsible for the difference in the soil bacterial communities detected in this study (Chapter 2 and Chapter 3). In addition, seasonal change can also directly affect the soil microbial communities through changes in temperature (Pietikainen et al. 2005) and soil water contents (Stevenson et al. 2014). Measuring these factors in future studies can potentially contribute significantly to our understanding of the changes detected in bacterial communities over the different seasons. The correlations observed in this study, between seasonal change and soil microbiota are of great concern when considering the potential implications of global warming. There is strong evidence that global warming effect plant distribution and physiology (Aryal, 2015; Prentice et al. 1992; Sykes et al. 2005) and this will inevitably influence soil microbial communities.

The observed seasonal effect on soil bacterial communities raised the question whether community function is affected. Microbial communities are known to be functionally redundant, which can be defined as the ability of a microbial taxon to perform a specific function at the same rate and environmental conditions as another taxon (Allison & Martiny, 2008). Whether this is the case for the different bacterial communities cannot be answered by results obtained in this study. Measuring microbial community function is a challenge and linking this to the microbes responsible has proven to be mostly impossible due to the sheer complexity of soil environments (Minz et al. 2013; Nesme et al. 2016). However, for future studies, a metatranscriptomics approach can be used to provide valuable information on microbial activity in the soil through investigating genes that are expressed at a specific time and environmental conditions. Combining metagenomics with metatranscriptomic will aid in our understanding of the structure of a specific community and the microbial activity, however, we will not be able to link specific groups of microbes to a specific function. Alternatively, as the cost of generating genome information is rapidly declining, whole genome sequencing (WGS) can also

be used in future studies (Van El et al. 2013). Data obtained from this technique can generate information on the structure as well as function of a community. Studies that have used WGS, investigated less complex environments such as food-associated environments (Stasiewicz et al. 2015) and health related studies (Van El et al. 2013). However, using WGS for environmental analysis as complex as soil still needs to be optimized as databases are incomplete.

Another aspect that adds to the complexity of soil ecosystems is the continuous interactions between microbes and their environment (Williams et al. 2014). It is often difficult to identify important interactions to study in complex soil environments. Therefore, we identified potential interactions based on Spearman correlations and visualized it through network analysis (Chapter 2) and a correlogram (Chapter 3). These correlations highlighted possible interactions between bacterial communities and the environment which can be used as the foundation for future studies. Of particular interest were the interactions of bacteria associated with nitrogen and phosphorus cycling. Bacterial species from Bradyrhizobiaceae and Burkholderiaceae are known to form root nodules with fynbos plants (Stepkowski et al. 2007; Verlag et al. 2002) and were identified to be part of a number of interactions in this study. The diversity and metabolic activities of root nodulating bacteria associated with *A. linearis* and *Cyclopia* spp. are mostly unexplored. In addition, the role other bacteria and environmental variables play on the function of these root nodules are unknown. Furthermore, some Pseudomonadaceae species are known to play a role in phosphate-solubilizing (Buch et al. 2008; Rodríguez et al. 2006) and members from this family were also identified to be part of a number of different interactions between other microbes and environmental variables. Fynbos soil are known to be P limiting and this group of bacteria may play an important role in the survival of these two herbal tea plant species. Very little is known on the diversity and role of Pseudomonadaceae species in the fynbos and to what extent this group of bacteria are involved in phosphate-solubilising.

Interactions between plant roots and fungi also play an important role in nitrogen fixation and phosphate-solubilisation (Alonso et al. 2008; Azcon et al. 1975; Chalot & Brun, 1998; Hodge & Fitter, 2010; Zhang et al. 2011). Although well studied in a variety of different environments, there are still a large number of undescribed root-

associated fungi. For instance, we found that the Helotiales, an order consisting of the most undescribed root associated species (Tedersoo et al. 2009), was one of the most dominant groups of fungi associated with rooibos plants (Chapter 4). Furthermore, it is hypothesized that fungi from the order Geoglossales are associated with specific plant hosts, but due to the lack in understanding the ecology of this group of fungi, potential plant host have not yet been established (Hustad et al. 2013; Loizides et al. 2015). In this study we provided evidence that the order Geoglossales might be associated with honeybush plants. Potential interactions between *Cyclopia* spp. and this fungal group are still unknown and future research might clarify some ecological questions of Geoglossales and its association with potential plant hosts.

Adding to the complexity to study soil environments, microbes not only interact with the environments, but also with each other. Of particular interest are the interactions between bacteria and fungi. Some bacterial species including members of the Pseudomonadaceae (e.g. *Pseudomonas fluorescens* and *Pseudomonas putida*) are known to have pathogenic interactions with certain soil fungi (Rainey et al. 1990). Furthermore, the inhibition of fungal spore germination by bacteria has also been reported. These spore-associated bacteria withdraw nutrients from the fungal spores, known as mycostasis, which results in the inhibition of spore germination (Lockwood, 1977; Lockwood, 1986; Toyota & Kimura, 1993).

Interactions between bacteria and fungi can also be mutualistic (De Boer et al. 2005; Kobayashi & Crouch, 2009). One of best known examples of this is the mutualistic interaction of ectomycorrhizal (EM) helper bacteria which have a positive effect of the establishment of EM fungi (Garbaye, 1994). It was reported that the association between specific nitrogen-fixing bacteria and the mycorrhizal fungus, *Rhizopogon*, contribute to the microaerophilic environments which are required for nitrogen fixation (Massicote & More, 1992). Interactions such as this can potentially play an important role in fynbos soil where nitrogen fixation by bacteria is crucial for the plants in this low nutrient environment. Other beneficial effects bacteria have on fungi may include the stimulation of arbuscular mycorrhizal (AM) growth (Barea et al. 2002; Budi et al. 1999; Medina et al. 2003) as well as increased formations of fruiting bodies, spore production and spore germination (Carpenter-Boggs et al. 1995; Mayo

et al. 1986). A better, more comprehensive understanding of the interactions between bacteria and fungi can aid in the development of potential biocontrol agents for fungal plant pathogens and assist with the stimulation of mycorrhizal infections (De Boer et al. 2005). With better knowledge on the composition of bacterial and fungal communities associated with *Aspalathus linearis* and *Cyclopia* spp., this study laid the foundation for future studies to investigate specific bacterial and fungal interactions which can potentially play an important role in the survival and adaption of fynbos in the CFR.

In conclusion, this study highlights the immense diversity and complexity of soil microbes within fynbos soil. Although we identified different bacterial and fungal taxa that may play a key role in the survival of *Aspalathus linearis* and *Cyclopia* spp. plants in the unique fynbos ecosystem, much more research is needed to elucidate on these complex interactions.

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Supplementary material

Chapter 2: Figures and Tables

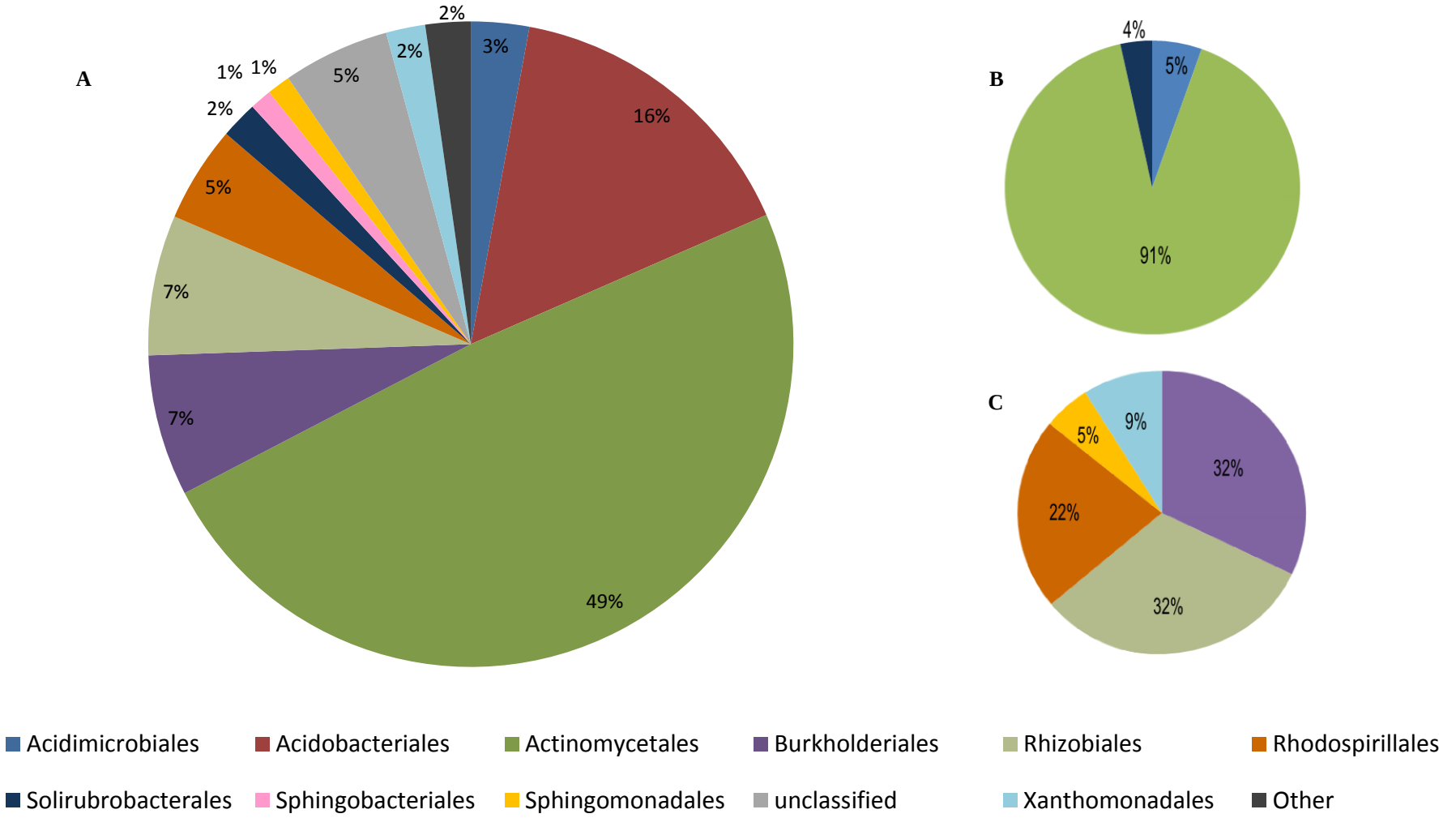

Figure S2.1: A.) Relative abundance of classified sequences at order level within all the samples. Sequences with a homology ≥ 80% were used and only orders with a mean relative abundance of ≥ 1% were included. B.) Relative abundance of orders belonging to the Actinobacteria phylum (Actinomycetales (91%), Acidinicrobiales (5%) and Solirubrobacterales (4%)) C.) Relative abundance of orders belonging to the Proteobacteria phylum (Burkholderiales (32%), Rhizobiales (32%), Rhodospirillales (22%), Sphingomonadales (5%) and Xanthomonadales (9%)).

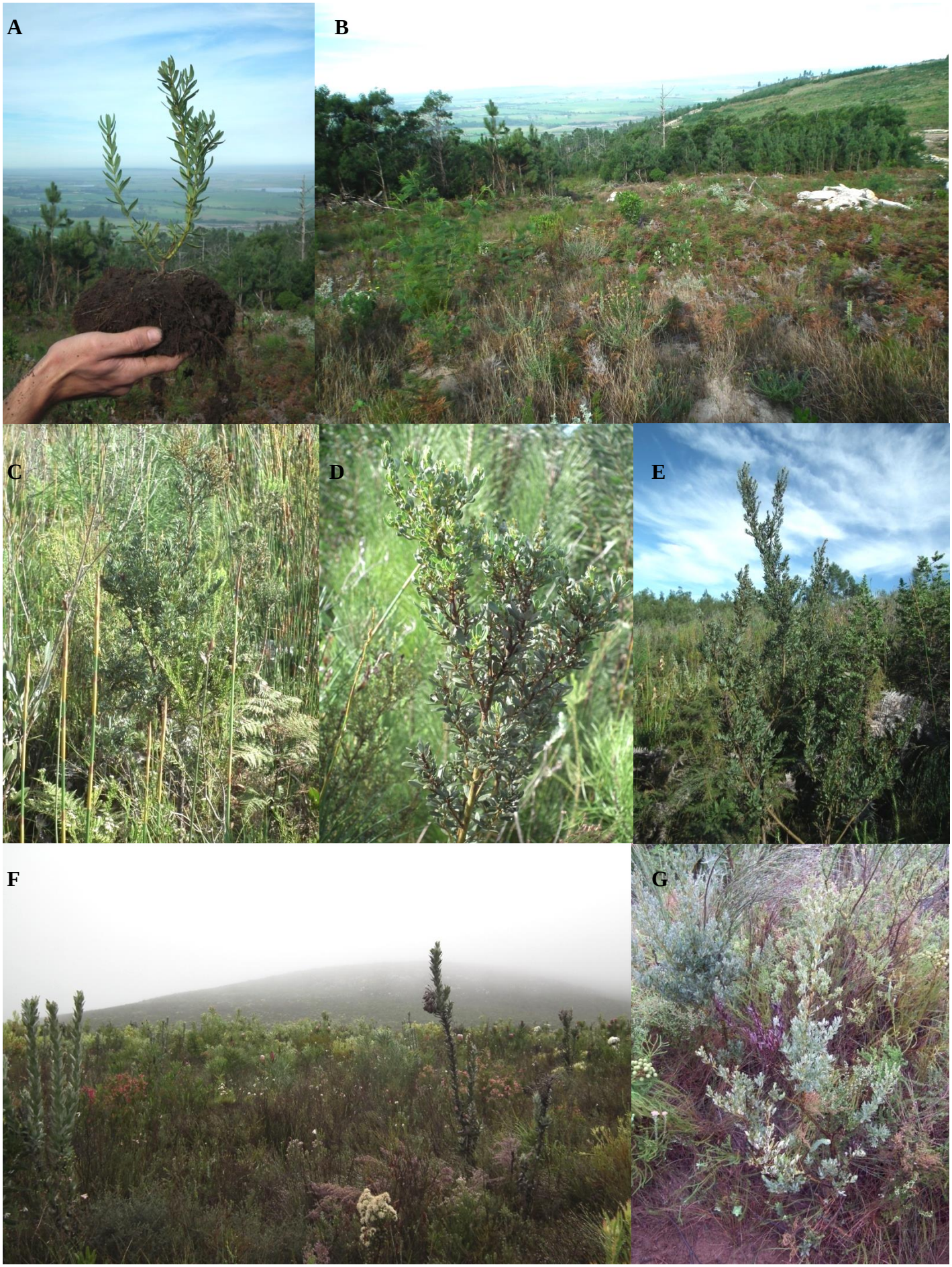

Figure S2.2: Some of the *Cyclopia* sp. sampling sites. An example of typical dense plant growth of commercial (B) and natural (F) sites. Some commercial plants sampled are shown in A and G, and some natural plants sampled shown in C, D and E.

Table S2.1: Barcode fusion PCR primers for 16S rRNA amplification (Lifetechnologies™, USA)

Primer name	Adaptor sequence	Barcode	Barcode adapter	Target forward
		Bacterial reverse		
B1R	-	-	-	5 -CTACCAGGGTATCTAATCCTG- 3'
		Bacterial forward		
B1F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTAAGGTAAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B2F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TAAGGAGAAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B3F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	AAGAGGATTC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B4F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TACCAAGATC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B5F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGAAGGAAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B6F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTGCAAGTTC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B7F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTCGTGATTC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B8F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTCCGATAAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B9F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TGAGCGGAAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B10F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTGACCGAAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B11F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCCTCGAATC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B12F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TAGGTGGTTC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B13F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTAACGGAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B14F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTGGAGTGTC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B15F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTAGAGGTC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B16F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTGGATGAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B17F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTATTCGTC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B18F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGGCAATTGC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B19F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTAGTCGGAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B20F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGATCCATC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B27F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	AACCATCCGC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B28F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATCCGGAATC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B29F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCGACCACTC	GAT	ACTCCTACGGGAGGCAGCAG- 3'

Table S2.2: Sequence processing

Sample number	Sample description (season, type and soil fraction)	Total reads	High quality reads	Non-chimeric unique reads
wcr1	wet, commercial and rhizosphere	147707	5478	5456
wcr2	wet, commercial and rhizosphere	36114	763	756
wcb1	wet, commercial and bulk soil	32630	616	613
wnr1	wet, natural and rhizosphere	45661	817	816
wnr2	wet, natural and rhizosphere	35475	818	818
wnb1	wet, natural and bulk soil	42017	742	740
wcr3	wet, commercial and rhizosphere	30997	409	409
wcr4	wet, commercial and rhizosphere	41776	666	662
wcb2	wet, commercial and bulk soil	30572	551	551
wnr3	wet, natural and rhizosphere	45284	764	760
wnr4	wet, natural and rhizosphere	42743	647	644
wnb2	wet, natural and bulk soil	23027	294	293
wcr5	wet, commercial and rhizosphere	46428	981	978
wcr6	wet, commercial and rhizosphere	85925	1792	1782
wcb3	wet, commercial and bulk soil	9341	222	222
wnr5	wet, natural and rhizosphere	39851	862	850
wnr6	wet, natural and rhizosphere	31660	613	611
wnb3	wet, natural and bulk soil	21746	508	508
dcr1	dry, commercial and rhizosphere	1248199	21400	20879
dcb1	dry, commercial and bulk soil	174505	3145	3134
dcr2	dry, commercial and rhizosphere	113783	2397	2376
dnr1	dry, natural and rhizosphere	101604	2211	2203
dnr2	dry, natural and rhizosphere	65163	1610	1604
dnb1	dry, natural and bulk soil	45511	1058	1058
dcr3	dry, commercial and rhizosphere	80976	1585	1582
dcb2	dry, commercial and bulk soil	117849	2339	2329
dcr4	dry, commercial and rhizosphere	87153	2027	2023
dnr3	dry, natural and rhizosphere	134826	2596	2593
dnr4	dry, natural and rhizosphere	100634	1943	1932
dnb2	dry, natural and bulk soil	134506	1868	1858
dnr5	dry, natural and rhizosphere	83546	1831	1825
dnr6	dry, natural and rhizosphere	132573	3263	3237
dcr5	dry, commercial and rhizosphere	93519	1908	1893
dcr6	dry, commercial and rhizosphere	80056	1482	1482
dcb3	dry, commercial and bulk soil	94065	1702	1692
dcr7	dry, commercial and rhizosphere	92035	1684	1675
dnr7	dry, natural and rhizosphere	254463	4870	4833
dnr8	dry, natural and rhizosphere	191707	4470	4451
dnb3	dry, natural and bulk soil	117205	2219	2203
dnr9	dry, natural and rhizosphere	105809	2168	2154
dnr10	dry, natural and rhizosphere	106724	2051	2038
Total		**4545365**	**89370**	**88523**

Table S2.3: P-values for chemical variables correlation with microbial communities

Chemical variables	p-value
pH	0.000999*
Resistance	0.002997*
H.	0.001998*
PBrayII	0.001998*
K	0.082917
Na.ec.	0.000999*
K.ec.	0.08991
Ca.ec.	0.158841
Mg.ec.	0.577423
C	0.000999*
NO3.N	0.002997*
NH4.N	0.080919
Na.bs.	0.616384
K.bs.	0.000999*
Ca.bs.	0.000999*
Mg.bs.	0.000999*

* significant correlations ($p<0.05$)

Supplementary material

Chapter 3: Figures and Tables

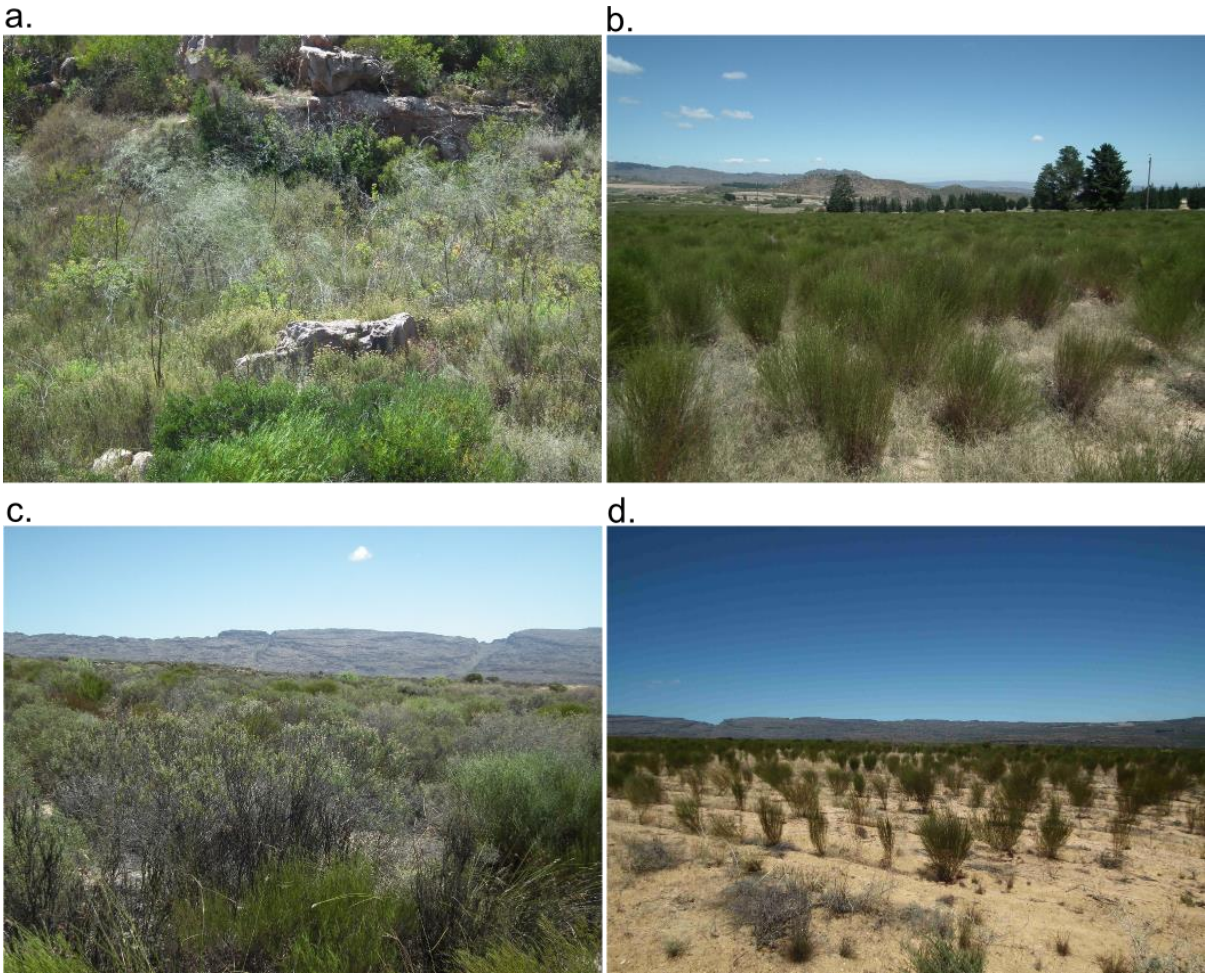

Figure S3.1: Representative photos of sampling sites on the different farms, Kleinvlei (a and b) and Klipopmekaar (c and d). Natural sites are characterized by dense fynbos vegetation (a and c), whereas commercial plants were easy accessible (b and d).

Table S3.1: Barcode fusion PCR primers for 16S rRNA amplification (Lifetechnologies™, USA)

Primer name	Adaptor sequence	Barcode	Barcode adapter	Target forward
		Bacterial reverse		
B1R	-	-	-	5 -CTACCAGGGTATCTAATCCTG- 3'
		Bacterial forward		
B1F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTAAGGTAAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B2F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TAAGGAGAAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B3F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	AAGAGGATTC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B4F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TACCAAGATC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B5F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGAAGGAAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B6F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTGCAAGTTC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B7F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTCGTGATTC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B8F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTCCGATAAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B9F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TGAGCGGAAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B10F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTGACCGAAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B11F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCCTCGAATC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B12F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TAGGTGGTTC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B13F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTAACGGAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B14F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTGGAGTGTC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B15F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTAGAGGTC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B16F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTGGATGAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B17F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTATTCGTC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B18F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGGCAATTGC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B19F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTAGTCGGAC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B20F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGATCCATC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B27F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	AACCATCCGC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B28F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATCCGGAATC	GAT	ACTCCTACGGGAGGCAGCAG- 3'
B29F	5' -CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCGACCACTC	GAT	ACTCCTACGGGAGGCAGCAG- 3'

Table S3.2: P-values for variables correlation with bacterial communities

Variables	p-value
Altitude	0.65035
Shannon	0.027972*
Chao	0.174825
pH	0.403596
Resistance	0.000999*
H	0.125874
P	0.545455
Na	0.000999*
K	0.000999*
Ca	0.689311
Mg	0.648352
C	0.035964*
+NO3	0.125874
NH4	0.063936
C/N	0.045954*
K/Na	0.372627

*significant correlations ($p<0.05$)

Table S3.3: Sequence processing

Sample number	Sample description	Total number of raw sequencing reads	High quality, non-chimeric unique reads
dcr1	Dry, commercial and rhizosphere soil	236259	2294
dcb1	Dry, commercial and bulk soil	448353	6355
dcr2	Dry, commercial and rhizosphere soil	238765	977
dcb2	Dry, commercial and bulk soil	368383	5425
dcr3	Dry, commercial and rhizosphere soil	210081	2799
dcb3	Dry, commercial and bulk soil	357884	5231
dnr1	Dry, natural and rhizosphere soil	270019	2217
dnr2	Dry, natural and rhizosphere soil	288208	3149
dnb1	Dry, natural and bulk soil	320984	4964
dcr4	Dry, commercial and rhizosphere soil	277759	4305
dcb4	Dry, commercial and bulk soil	398525	6521
dnr3	Dry, natural and rhizosphere soil	292092	2997
dnb2	Dry, natural and bulk soil	413189	4732
dcb5	Dry, commercial and bulk soil	402996	6512
dcr5	Dry, commercial and rhizosphere soil	307270	2490
dcr6	Dry, commercial and rhizosphere soil	268705	5070
dnr4	Dry, natural and rhizosphere soil	359280	2981
dnr5	Dry, natural and rhizosphere soil	487728	5211
dnr6	Dry, natural and rhizosphere soil	214733	3065
dnr7	Dry, natural and rhizosphere soil	202115	2217
wcb1	Wet, commercial and bulk soil	188735	3738
wcr1	Wet, commercial and rhizosphere soil	214221	5262
wcr2	Wet, commercial and rhizosphere soil	163116	3637
wnr1	Wet, natural and rhizosphere soil	234981	6140
wnr2	Wet, natural and rhizosphere soil	135724	3688
wnb1	Wet, natural and bulk soil	316362	5178
wnr3	Wet, natural and rhizosphere soil	1188164	38118
wcr3	Wet, commercial and rhizosphere soil	144060	3728
wcr4	Wet, commercial and rhizosphere soil	99910	2256
wcb2	Wet, commercial and bulk soil	138910	2340
wcb3	Wet, commercial and bulk soil	123199	2592
wcb4	Wet, commercial and bulk soil	141317	2420
wcr5	Wet, commercial and rhizosphere soil	100590	3109
wcb5	Wet, commercial and bulk soil	149818	3875
wcr6	Wet, commercial and rhizosphere soil	158538	2737
wcb6	Wet, commercial and bulk soil	234012	3765
wnr4	Wet, natural and rhizosphere soil	132647	6316
wnr5	Wet, natural and rhizosphere soil	56335	1369
wnb2	Wet, natural and bulk soil	110148	1504
wnr6	Wet, natural and rhizosphere soil	98218	2230
wnr7	Wet, natural and rhizosphere soil	110818	2423
wcb7	Wet, commercial and bulk soil	156926	3196
Total		**10760077**	**189133**

Table S3.4: Significant p-values for correlations between bacterial taxa and soil chemical variables

Var1	Var2	r	p
C	C/N	0.993009388	0
Na	K	0.898806214	6.66E-16
Ca	Mg	0.867762506	1.02E-13
C/N	Mg	0.866199672	1.27E-13
C	Mg	0.864434838	1.63E-13
Shannon	Chao	0.775066078	1.69E-09
Ca	C/N	0.757004201	6.59E-09
Ca	C	0.745503664	1.48E-08
Deltaproteobacteria	unclassified	0.69674027	0.000000297
Resistance	K	-0.680337668	0.000000718
Resistance	Na	-0.662911356	0.00000172
Kineosporiaceae	Nocardioidaceae	0.630365193	0.00000768
Geodermatophilaceae	Nocardioidaceae	0.607931018	0.0000195
Geodermatophilaceae	Kineosporiaceae	0.605950117	0.0000211
Resistance	H	-0.561879814	0.000108
K	NH4	0.560951114	0.000111
Pseudomonadaceae	K	0.544068873	0.000196
Gemmatimonadaceae	unclassified	0.541145027	0.000215
Gemmatimonadaceae	Deltaproteobacteria	0.536861002	0.000247
H	C	0.531128049	0.000296
Sporichthyaceae	unclassified	0.525606632	0.000351
H	Mg	0.522738814	0.000383
Resistance	Sporichthyaceae	0.513764083	0.000501
Resistance	NO3	-0.508591771	0.000584
K	NO3	0.50780046	0.000597
Geodermatophilaceae	Bradyrhizobiaceae	-0.507739544	0.000598
Gemmatimonadaceae	Sporichthyaceae	0.503388226	0.000678
H	C/N	0.5014292	0.000718
Geodermatophilaceae	Shannon	0.492770881	0.000915
Pseudomonadaceae	Na	0.490184397	0.000983
Bradyrhizobiaceae	C	0.484894782	0.00114
Resistance	Mg	-0.4800632	0.00129
Bradyrhizobiaceae	C/N	0.479543984	0.00131
Deltaproteobacteria	Sporichthyaceae	0.476572573	0.00142
Resistance	Sporolactobacillaceae	0.471131414	0.00164
Mycobacteriaceae	Altitude	-0.465818107	0.00188
pH	Ca	0.460484177	0.00215
Na	NH4	0.443160921	0.00329
Kineosporiaceae	Gemmatimonadaceae	0.441430122	0.00343
C/N	K/Na	-0.437123597	0.0038
Bradyrhizobiaceae	Rhodocyclaceae	0.435265869	0.00397
H	pH	-0.416903496	0.00602
H	Na	0.414620727	0.00633
Kineosporiaceae	Shannon	0.410456002	0.00694
Geodermatophilaceae	NH4	-0.410286129	0.00696
Xanthomonadaceae	Bradyrhizobiaceae	0.400573224	0.00857
C	K/Na	-0.399739921	0.00872
Sporolactobacillaceae	Mg	-0.395291835	0.00957
Mycobacteriaceae	pH	0.395094276	0.00961
NO3	K/Na	0.389948219	0.0107

Pseudomonadaceae	Resistance	-0.389244378	0.0108
unclassified	C/N	0.387730986	0.0112
Nocardioidaceae	Bradyrhizobiaceae	-0.384674013	0.0119
Nocardioidaceae	Shannon	0.376224726	0.0141
unclassified	K	-0.374394178	0.0146
unclassified	C	0.371642739	0.0154
Bradyrhizobiaceae	Sporolactobacillaceae	0.367173851	0.0168
Sporichthyaceae	K	-0.366803259	0.0169
H	NH4	0.365551531	0.0173
Mycobacteriaceae	H	-0.36337477	0.018
Gemmatimonadaceae	Resistance	0.358340323	0.0198
Resistance	Deltaproteobacteria	0.354270399	0.0213
Gemmatimonadaceae	Nocardioidaceae	0.353128821	0.0218
Rhodocyclaceae	C/N	0.353150189	0.0218
Na	NO3	0.349896729	0.0231
Sporichthyaceae	Sporolactobacillaceae	0.34938708	0.0233
Bradyrhizobiaceae	K	-0.346009344	0.0248
Rhodocyclaceae	C	0.343014717	0.0262
Ca	K	-0.342380852	0.0265
Kineosporiaceae	Bradyrhizobiaceae	-0.340653122	0.0273
Geodermatophilaceae	Xanthomonadaceae	-0.337397784	0.0289
Mycobacteriaceae	Resistance	0.332176805	0.0316
H	Sporichthyaceae	-0.327317566	0.0344
Deltaproteobacteria	K	-0.326694101	0.0347
Resistance	NH4	-0.325882107	0.0352
Sporichthyaceae	Na	-0.321362674	0.038
Bradyrhizobiaceae	NO3	-0.320413619	0.0386
H	Sporolactobacillaceae	-0.317816615	0.0403
Resistance	C	-0.317314327	0.0406
Na	K/Na	-0.315535188	0.0418
Geodermatophilaceae	Sporolactobacillaceae	-0.305431396	0.0492

□ Boxed values indicate negative correlations

Supplementary material

Chapter 4: Figures and Tables

Table S4.1: Unique barcodes used to label the forward primer (ITS1f)

Name	Plant	Type	Barcode name	Barcode
rc8c	Rooibos	Commercial	IonXpress_007	TGCCACGAAC
rc3b	Rooibos	Commercial	IonXpress_002	CAGATCCATC
rc8b	Rooibos	Commercial	IonXpress_006	TTCGAGACGC
rc7a	Rooibos	Commercial	IonXpress_003	CTCGCAATTA
rc10a	Rooibos	Commercial	IonXpress_001	TCTGGATGAC
rc3a	Rooibos	Commercial	IonXpress_069	TTAGTCGGAC
rn2c	Rooibos	Natural	IonXpress_009	AGGCAATTGC
rn9d	Rooibos	Natural	IonXpress_008	CCTGAGATAC
rn9c	Rooibos	Natural	IonXpress_049	AACCATCCGC
rn2b	Rooibos	Natural	IonXpress_004	TCTATTCGTC
rn9a	Rooibos	Natural	IonXpress_064	AACCTCATTC
rn9b	Rooibos	Natural	IonXpress_012	TTACAACCTC
hc4c	Honeybush	Commercial	IonXpress_057	TGAGCGGAAC
hc1a	Honeybush	Commercial	IonXpress_059	CTAAGGTAAC
hc4b	Honeybush	Commercial	IonXpress_056	TTCCGATAAC
hc6a	Honeybush	Commercial	IonXpress_005	TCTAACGGAC
hc6c	Honeybush	Commercial	IonXpress_011	TTCGTGATTC
hc1f	Honeybush	Commercial	IonXpress_047	AAGAGGATTC
hc1d	Honeybush	Commercial	IonXpress_060	TAAGGAGAAC
hn2a	Honeybush	Natural	IonXpress_010	TACCAAGATC
hn2c	Honeybush	Natural	IonXpress_048	CTGCAAGTTC
hn5a	Honeybush	Natural	IonXpress_013	CTGACCGAAC
hn7c	Honeybush	Natural	IonXpress_063	TCTAGAGGTC
hn7b	Honeybush	Natural	IonXpress_062	TTGGAGTGTC
hn2b	Honeybush	Natural	IonXpress_061	CAGAAGGAAC
hn5c	Honeybush	Natural	IonXpress_058	TAGGTGGTTC

Table S4.2: Significant P-values for chemical variables correlation with fungal communities

Chemical variables	p-value
K	0.04799
NO3	0.027594
Mg	0.011998

Table S4.3: Fungal orders associated with either honeybush or rooibos plants only

Plant	Fungal order	% Relative abundance
Honeybush	Atheliales	0.31
	Boletales	2.10
	Chaetosphaeriales *	1.06
	Geoglossales *	2.92
	Hymenochaetales	0.44
	Pezizales	0.18
	Russulales *	2.92
Rooibos	Coniochaetales *	1.12
	Eurotiomycetes_unidentified	0.11
	Onygenales	0.13
	Ophiostomatales	0.24
	Polyporales	0.35
	Saccharomycetales	0.19
	Spizellomycetales	0.65

* $\geq$ 1 % relative abundance